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THE UNIVERSITY OF ALBERTA

IN VITRO AND IN VIVO METABOLISM
AFTER SUB-TOTAL NEPHRECTOMY

by



UWE KURT TURNER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

IN

BIONUCLEONICS

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

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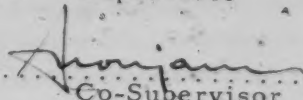
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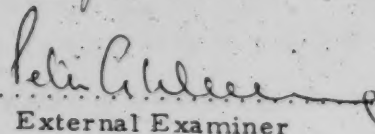
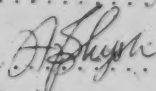
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After Sub-Total Nephrectomy submitted by Uwe Kurt Turner
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy in Bionucleonics.



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DEDICATION

To Gail and Lance,

who made it all worthwhile.

ABSTRACT

The influence of subtotal nephrectomy in rats, on in vivo and in vitro metabolism, has been investigated.

In the in vivo studies, ^{14}C -Vitamin D_3 and ^3H -25-Hydroxy Vitamin D_3 were administered both intraperitoneally and intravenously, in separate experiments. The distribution of these hormones and their metabolites, was determined in plasma and various other tissues. It was observed that uremic animals exhibited significantly higher levels of radioactivity in the blood, representing circulating ^{14}C -Vitamin D_3 and its metabolites, than the levels found in control rat blood. Chromatographic analysis of the lipid extracts of plasma, obtained from uremic rats, revealed the presence of significant quantities of metabolites more polar than 25-Hydroxy Vitamin D_3 . These metabolites were not readily observed in control rat plasma extracts. These results give support to the idea that, in uremia, the rate of metabolism of Vitamin D_3 is accelerated.

The in vivo studies were followed by a series of in vitro studies. The first in vitro investigations were performed to examine the rate of hydroxylation of labeled Vitamin D_3 by liver homogenates obtained from control, rachitic, and uremic rats. The results suggested that there was no significant difference in the rate of hydroxylation of Vitamin D_3 , under the experimental conditions, in the various groups of rat livers.

Examination of the in vitro rate of metabolism of 25-Hydroxy Vitamin D_3 by control and uremic rat renal homogenate preparations revealed that the rate of hydroxylation of this hormone was significantly reduced in uremic rat renal mitochondria.

Assay of the hepatic microsomal enzyme activity in normal and uremic rats, showed that there was a significant change in the activities of oxidative and reductive drug metabolizing enzymes. Sub-totally nephrectomized rats demonstrated a significant reduction in the activity of hexobarbital oxidase, aminopyrine N-demethylase, as well as p-nitrobenzoic acid reductase. This was accompanied by a concomitant reduction in cytochrome P-450.

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I. INTRODUCTION

Chronic renal failure in man can lead to numerous clinical complications as a result of the attending metabolic acidosis, intestinal malabsorption of both amino acids and minerals, increased circulating levels of parathyroid hormone (PTH) and uremic toxins such as indoles and guanidines. These biological derangements, either individually or acting together have a profound effect on the various homeostatic mechanisms in the body.

Alterations in the metabolic fate of carbohydrates have been well documented in both uremic man and in animals with experimental renal failure (50-54). There is an altered uptake of glucose by body tissues, as well as changes in the various carbohydrate metabolic pathways. This is also associated with altered levels of circulating insulin which appear to be reduced at that time.

Alterations in lipid metabolism have also been demonstrated in uremia, manifested as hyperlipemia, primarily hypertriglyceridemia (55,56). These changes have been attributed to an abnormal carbohydrate metabolism, such as increased use of the pentose shunt. Increase in hepatic synthesis has also been implicated along with a simultaneous decrease of lipoprotein lipase.

Protein metabolism is also significantly altered in the uremic syndrome (57,58). There is evidence of increased protein synthesis by the liver, as well as increased production of urea by the uremic liver (59).

A number of other changes have also been described in the uremic syndrome. The anemia found in uremia is partly due to decreased production of erythropoietin by the renal tissues. The increased hemolysis appears to be due to a direct effect of the uremic toxins on the erythrocyte cell membrane. The coagulation defect observed in this syndrome appears to be due to a thrombocytic defect. This has been attributed

to a decrease in activity of Factor III in thrombocytes (96). Furthermore a thrombocytopenia has also been demonstrated.

Many of these abnormalities in uremia can often be improved by dialysis. In contrast, the decreased intestinal absorption of calcium is usually unaffected by dialysis. Progressive renal osteodystrophy and demineralization of bone is also observed in dialyzed patients despite the reversal of hypocalcemia, hyperphosphatemia, and acidosis, to normal levels. The osteodystrophy, with its accompanying defect in maturation of both the bone collagen and mineral, can be variably attributed to hyperparathyroidism, chronic acidosis, calcium malabsorption, and especially defective synthesis of the biologically active form of Vitamin D₃.

It has been shown that in uremia, there is an accelerated fractional turnover of Vitamin D₃, as well as an abnormal accumulation of biologically inactive metabolites (44). Furthermore the activity of various drug metabolizing enzymes have been shown to be altered in uremic patients to varying degrees (97).

The understanding of some of the basic mechanisms involved in the alteration of Vitamin D metabolism and changes in the metabolism of therapeutic agents in uremic individuals is vital to effective management of these patients. The purpose of this investigation is therefore twofold:

1. to study some of the metabolic pathways involved in drug metabolism and detoxification by hepatic microsomal enzymes in vitro in control and uremic rats, and,
2. to examine the metabolic pattern of Vitamin D, both in vivo and in vitro, in control and uremic animals, and thus attempt to gain some insight into the pathophysiology of uremia.

II. LITERATURE SURVEY

A. Vitamin D

1. Historical

Calciferol has been recognized as a vitamin for forty years. However, it is only recently that significant studies have been made in relation to the function and the metabolism of Vitamin D.

In 1919, Mellanby (48) demonstrated that he could cure rickets in dogs by the administration of cod liver oil, thereby proving that rickets was a nutritional disease. In 1922, McCollum *et al.* (99) demonstrated that the antirachetic activity of cod liver oil was due to a heat resistant substance which McCollum called Vitamin D.

The work of Steenbock and co-workers (100) demonstrated that antirachetic activity could be induced in foods and in animals by ultraviolet (uv) irradiation. The demonstration that the uv activated material was in the sterol fraction led to the isolation and identification of Vitamin D₂ (ergocalciferol) by Askew *et al.* (101) and Windaus *et al.* (102). For a time it was assumed that this was the sole Vitamin D. Further work demonstrated that Vitamin D could also be produced from cholesterol (103) and Windaus *et al.* (104) isolated and identified Vitamin D₃ (cholecalciferol).

The understanding of Vitamin D metabolism was hampered by the fact that in the pure form, it was an extremely potent agent. The quantity to which an animal responded was extremely small (1 I. U. = 0.025 μ g). Inadequate detection techniques blocked further understanding of the biochemical behavior of Vitamin D. It was not until Kodicek used radioactively labeled Vitamin D that significant progress was made in the study of the metabolism of Vitamin D (105). However, the specific activity of the labeled material was quite low and the substance still had to be used in massive non-physiological doses. It was not until the early 1960's that ³H-Vitamin D of high specific activity was produced by using the Wilzbach technique (106) in an exchange reaction with the pro-vitamin, and then converting the product to Vitamin D₃ by

ultraviolet irradiation (107,108). Now with the advent of Vitamin D of high specific activity, significant progress has been made in understanding the biochemical role of Vitamin D₃. The radioactive label made possible the detection of Vitamin D metabolites as well as providing a sensitive method for the detection of Vitamin D itself, especially when administered in physiological doses.

Two other techniques which advanced the understanding of Vitamin D metabolism were the development of effective extraction methods for lipids and more sophisticated chromatographic techniques. The method of Blight and Dyer (109), involving the use of chloroform and methanol, proved to be effective in quantitative extraction of lipids. Advances in chromatography of lipids also aided in the acceleration of understanding of Vitamin D metabolism. Silicic acid column chromatography (110) as well as thin layer chromatography (111) shed new light on the biochemistry of Vitamin D. The utilization of Sephadex LH-20 (112) finally helped to unravel the mystery of this substance.

2. Metabolism of Vitamin D and Isolation of the Metabolites

Early investigations with either unlabeled or labeled Vitamin D₃ indicated that the primary site of absorption was the ileum (103) and that bile was important in this process. Later work by Schachter et al. (114) provided evidence that Vitamin D was mainly absorbed by the jejunum. However, more recent work by Rosenstreich et al. (113) indicated that Vitamin D is absorbed throughout the intestinal tract, depending on the state of the Vitamin D balance in the animal (115).

Once Vitamin D was absorbed, it entered the lymphatic system, where most of it traveled in the chylomicron fraction (114). Once in the blood stream, it became associated with the α_2 globulin fraction (117). The rate of absorption of Vitamin D was also a function of the level of concentration of the Vitamin D in the body, especially that of the storage sites. Vitamin D deficient rats, as well as humans, were able to absorb more Vitamin D than animals or people pretreated with non-radioactive Vitamin D (115).

Storage of Vitamin D and its metabolites occurred largely in the fat deposits throughout the body (113), and not primarily in the liver as was believed earlier (116). Vitamin D and its metabolites seemed to concentrate in almost all tissues which contained lipid, including muscle and bone.

Excretion of Vitamin D and its metabolites occurred primarily through the biliary system into the small intestine (108). As much as 30% of an administered dose appeared in the bile, within 24 to 48 hours after a single infusion of labeled Vitamin D (108); whereas only 2% of the dose normally appeared in the urine. The main form of the vitamin excreted in the bile appeared to be the glucuronide conjugates of Vitamin D₃ and its metabolites. A sulphate ester has been identified in both milk and urine.

a. Biotransformation of Vitamin D

A major breakthrough in Vitamin D biochemistry occurred with the advent of labeled Vitamin D of high specific activity. Administration of ³H-Vitamin D₃ in physiological doses to rats revealed that Vitamin D was metabolized to biologically active metabolites. Norman et al. (118) and Lund and DeLuca (110) were able to demonstrate, by means of chloroform-methanol extraction of tissues followed by silicic acid chromatography, that there were at least three metabolites present in rat kidney, intestine, liver, blood, bone and feces. The first group of metabolites in the chromatographic profile was conclusively demonstrated to be primarily esters of Vitamin D and long chain fatty acids (119). The second metabolite, somewhat less polar than the parent compound, was biologically active, but has not yet been identified. The fourth peak eluted, more polar than Vitamin D, had a biological activity at least equal to the parent compound (110). The quantity of peak IV appearing in the serum increased as the dose of labeled Vitamin D decreased, indicating that there was a possible product inhibition effect (110). This compound acted more rapidly in initiating calcium transport by everted gut sacs (120), as well as in initiating bone resorption.

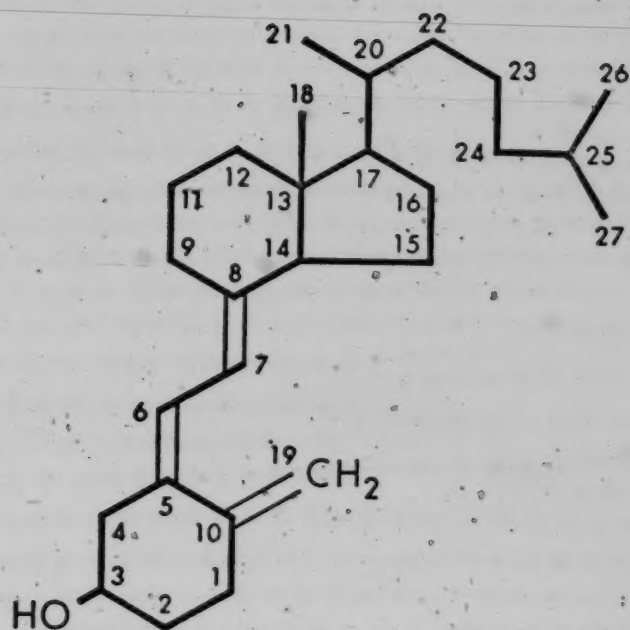


Figure 1. Nomenclature of the Structure of Vitamin D₃

Isolation and identification of the more active metabolite of Vitamin D occurred in 1968 when Blunt *et al.* (121) isolated a large quantity of the metabolite from the plasma of several hogs which had been fed ^3H -labeled Vitamin D for 25 days. The compound was characterized and unequivocally identified to be 25-hydroxy-Vitamin D_3 $25(\text{OH})\text{D}_3$. It was shown by Ponchon and DeLuca (122, 123) that this compound was formed primarily in the liver. This metabolite has proved to be the major circulating metabolite of Vitamin D in the blood (124, 125). Horsting and DeLuca (124, 125) demonstrated an enzyme system in the liver, requiring oxygen and reduced pyridine nucleotide, which was responsible for the metabolic conversion of Vitamin D.

In perfused intestine (126) and in bone culture (18) $25(\text{OH})\text{-D}_3$ proved to be active in transporting and mobilizing calcium in these end organs respectively; whereas Vitamin D was inactive. These findings, plus the fact that the $25(\text{OH})\text{-D}_3$ acted more rapidly *in vivo* suggested that 25-hydroxylation was essential for Vitamin D action.

b. Identification of Metabolites More Polar Than $25(\text{OH})\text{-Vitamin D}_3$

The synthesis of $25(\text{OH})\text{-D}_3$ (127) made possible the production of the compound labeled at carbon 26 and carbon 27, with tritium. This labeled metabolite was then used by Cousins *et al.* (128) to see whether it acted directly on target tissue or whether it underwent further biochemical transformations. Cousins *et al.* (129) observed that the $25(\text{OH})\text{-D}_3$ was rapidly accumulated by intestinal mucosal nuclei, then removed rapidly, accompanied by the appearance of more polar metabolites. As early as 1968, Haussler, Myrtle and Norman (130) found a polar metabolite, which they called 4B, in the intestine of animals given radioactive Vitamin D_3 . Later, they tested this metabolite for biological activity and observed that it had a very marked

intestinal calcium transport activity (131). Lawson *et al.* (132) were able to demonstrate a metabolite formed in chick intestine which had lost the C-1- ^3H , when they were given dual labeled (^{14}C and ^3H)-Vitamin D; they referred to this metabolite as peak 'P'. A similar metabolite was also found by Panchon and DeLuca (133) in both bone and small intestine; however, there seemed to be little biochemical activity, partly because the silicic acid chromatography failed to resolve the various metabolic components of $25(\text{OH})\text{-D}_3$ adequately. This led to the development of a new method of gel-liquid partition chromatography using Sephadex LH-20[®], an isopropyl ether derivative of Sephadex G-25[®] (112). This procedure allowed for the rapid resolution of all the 'peak V' metabolites, and also provided nearly 100% recovery of applied material. Continued work in this area resulted in the isolation and identification of the metabolite responsible for active transport of calcium in the intestine. Early in 1971, 1,25-dihydroxy-Vitamin D₃ [$1,25(\text{OH})_2\text{D}_3$] was identified (134-136). It was shown that this metabolite was also responsible for the mobilization of calcium from the skeleton (20, 21). Lawson *et al.* (134) were the first to demonstrate that this metabolite was produced by the kidney both *in vivo* and *in vitro*. This was confirmed by other workers (137, 138).

A more detailed analysis of the metabolic 'peak V' region of Vitamin D in plasma resulted in the finding of at least three different metabolic peaks. The first peak investigated was Va, which was the least polar of the group. To achieve the isolation of an adequate quantity of the metabolite for identification purposes, eight pigs were given large doses of Vitamin D₃ for 28 days. The peak V region was isolated by silicic acid chromatography. This peak was then rechromatographed using a silicic acid column and the peak Va was isolated. It was then rechromatographed on celite and then on

Sephadex LH-20. The pure metabolite was then identified as 21,25-dihydroxy-Vitamin D_3 [$21,25(OH)_2D_3$]. It was shown to have a marked effect on the mobilization of bone mineral while having a small but significant effect on intestinal calcium transport (139).

It was shown that this metabolite was made exclusively by the kidney (140,141). Later, it was established that this metabolite was in fact 24,25-dihydroxy-Vitamin D_3 [$24,25(OH)_2D_3$] and not $21,25(OH)_2D_3$, as reported earlier (142).

A further metabolite was isolated similarly as described for $24,25(OH)_2D_3$, namely peak Vc (143). It has been identified as 25,26-dihydroxy-Vitamin D_3 [$25,26(OH)_2D_3$]. It has also been demonstrated in plasma of chicks and rats given physiological doses of the vitamin. This metabolite has been shown to have some activity in initiating intestinal calcium transport but to have virtually no effect on mobilizing bone mineral. The organ which was responsible for the production of this metabolite has not yet been identified.

Further investigation of the biological activity and metabolism of $24,25(OH)_2D_3$ showed that there was a marked increase in intestinal calcium transport and serum calcium concentration of Vitamin D deficient rats fed a diet containing adequate calcium and phosphorus. These responses were observed to occur after a considerable time lag following the administration of the metabolite. Radioactively labeled $24,25(OH)_2D_3$, given intravenously to rats, was found to be metabolized to a more polar compound which increased in concentration in serum, intestinal mucosa and bone over a 48 hour period. Nephrectomy, or feeding a high calcium diet, abolished the stimulation of intestinal calcium transport by $24,25(OH)_2D_3$ and the *in vivo* production of the more polar compound from tritiated $24,25(OH)_2D_3$. This metabolite, identified as 1,24,25-trihydroxy-Vitamin D_3 [$1,24,25(OH)_3D_3$], having been produced by the kidney (144) facilitated calcium absorption without stimulating bone calcium mobilization.

3. Factors Regulating the Production of Metabolites of Vitamin D

a. Regulation of 25-hydroxylase Activity by the Liver

The liver has been shown to be the only site for the hydroxylation of Vitamin D at the 25-C position (122,123). In in vitro studies using liver homogenates, the 25-hydroxylation step occurred requiring some form of reduced pyridine nucleotide and molecular oxygen (125). Further work indicated that the hydroxylation occurred in the liver microsomal fraction (145). These studies revealed that liver homogenates prepared from animals given a prior dose of Vitamin D, hydroxylated Vitamin D at the 25-C position very slowly (125). The inhibition of 25-hydroxylase activity has appeared somewhat complex, as it was demonstrated that this reduced activity occurred within three hours after administration of Vitamin D to Vitamin D deficient rats (125). It also appeared that $25(\text{OH})_2\text{D}_3$ pretreatment had little effect on the reduction of 25-hydroxylase activity. In contrast, liver homogenates hydroxylated dihydrotachysterol equally well whether or not the rats had been pretreated with Vitamin D. Also, the production of the 25-hydroxy metabolite of dihydrotachysterol was proportionate to the amount administered in vivo (146), whereas the rate of appearance of $25(\text{OH})\text{D}_3$ in blood increased insignificantly, even if large doses of the parent compound were administered (146). While the exact mechanism by which the 25-hydroxylase enzyme system was turned off was not really understood, it has been shown to serve to control the rate of production of the circulating metabolite, $25(\text{OH})\text{D}_3$ thus protecting against possible Vitamin D toxicity.

b. Regulation of $25(\text{OH})\text{D}_3$ -hydroxylase Activity in the Kidney

Early work by a number of workers (147,148) demonstrated that there was an inverse relationship between the concentration of calcium in the diet and the efficiency of calcium absorption by the intestine; animals fed a low calcium diet adapted by increasing their

efficiency of intestinal absorption, while animals fed a high calcium diet adapted by decreasing their efficiency of calcium absorption. Nicolaysen and his group (148) felt that since intestine calcium absorption seemed to be related to the amount of bone calcification required, an endogenous factor must have been secreted by the skeleton which would inform the intestine of the skeletal needs for calcium.

The discovery by Kodicek and his group (149) that the kidney was the sole site for activation of $25(\text{OH})\text{D}_3$ to $1,25(\text{OH})_2\text{D}_3$ led to further investigations by many workers on how the synthesis of this highly active metabolite was regulated, and thereby regulating the calcium transfer by the gut and bone. Boyle *et al.* (150) reasoned that since dietary calcium had an effect on the efficiency of calcium absorption by the gut, this may also have modulated the synthesis of $1,25(\text{OH})_2\text{D}_3$. Boyle *et al.* therefore examined the effect that high and low calcium diets had on the *in vivo* metabolism of ^3H - $25(\text{OH})\text{D}_3$ in Vitamin D-deficient rats. They observed that dietary calcium concentration as well as serum calcium had a profound effect on the production of active metabolite $1,25(\text{OH})_2\text{D}_3$ as well as on the production of $24,25(\text{OH})_2\text{D}_3$. It was shown when the plasma concentration in Vitamin D deficient rats was low, the role of *in vivo* conversion of $25(\text{OH})\text{D}_3$ to $1,25(\text{OH})_2\text{D}_3$ was increased, while the metabolism to $24,25(\text{OH})_2\text{D}_3$ was decreased. A higher serum calcium concentration resulted in the reduction of $1,25(\text{OH})_2\text{D}_3$ while the quantity of $24,25(\text{OH})_2\text{D}_3$ was increased. The change from the production of one metabolite to the other was abrupt, rather than gradually occurring at a serum calcium concentration of 9.0-9.6mg/100ml (150).

Omdahl and DeLuca (39, 40) while investigating rachetogenic activity of dietary strontium, were able to show in both *in vivo* and *in vitro* experiments that strontium treated animals, or renal mitochondria, produced $24,25(\text{OH})_2\text{D}_3$ and very little $1,25(\text{OH})_2\text{D}_3$. The question then arose, whether the mechanism of the modulation of

$25(\text{OH})\text{D}_3$ metabolism was regulated by direct action of serum calcium on the kidney or whether humeral factors released in response to the serum calcium acted on the kidney.

Boyle *et al.* examined the effect produced by the addition of calcium to a mitochondrial suspension from chick kidney, and found that it did not appreciably reduce the rate of 1-hydroxylation of $25(\text{OH})\text{D}_3$. Furthermore, they found that when animals were shifted from low calcium diets to diets of a higher calcium content, there was a time lag of several days before the activity of the 1-hydroxylation system was reduced and the 24-hydroxylation was initiated (150). These findings were suggestive of regulation by hormones such as PTH or calcitonin which would be sensitive to serum calcium concentration, rather than serum calcium concentration *per se*.

Rasmussen *et al.* (151) demonstrated that PTH and calcitonin did influence the metabolic rate of $25(\text{OH})\text{D}_3$. These workers observed that the addition of PTH to an incubate of isolated chick renal tubules and $25(\text{OH})\text{D}_3$ stimulated $1,25(\text{OH})_2\text{D}_3$ synthesis. In contrast, the addition of calcitonin to the incubate inhibited $1,25(\text{OH})_2\text{D}_3$ production by the renal tubules. These results were analogous to the *in vivo* situation where, under hypocalcemic conditions, there was an elevated level of PTH and a reduced calcitonin, while in the hypercalcemic or normo-calcemic physiological state there was an elevated level of calcitonin and a reduced serum concentration of PTH. This appeared to support the theory that parathyroid hormone may work as a trophic hormone for the synthesis of the $1,25(\text{OH})_2\text{D}_3$. The inter-relationship of parathyroid hormone and serum calcium *in vivo* was examined by regulating the synthesis of $1,25(\text{OH})_2\text{D}_3$ (152). Garabedian *et al.* (152) studied the influence of thyro-parathyroidectomy in animals on a low calcium diet, with their ability to metabolize $25(\text{OH})\text{D}_3$. Animals on a low calcium diet made predominantly $1,25(\text{OH})_2\text{D}_3$ until after thyro-parathyroidectomy, when this ability was found to be completely

lost and they made $24,25(\text{OH})_2\text{D}_3$. The complete transition took place 48 hours post-operatively. Administration of PTH extract every 6 hours to these animals restored the 1-hydroxylation to pre-operative levels, while 24-hydroxylation activity was reduced to pre-operative levels. Work carried out by Fraser and Kodicek (153) using chickens as the animal model supported these findings. MacIntyre's group (154,155) studied the influence of large doses of PTH and calcitonin on the metabolism of $25(\text{OH})\text{D}_3$ in intact rats. They observed that PTH administration resulted in the production of $24,25(\text{OH})_2\text{D}_3$ and the inhibition of $1,25(\text{OH})_2\text{D}_3$ synthesis, whereas large doses of calcitonin had the opposite effect. These findings were contrary to those of other workers (153). The role that the calcium and phosphate ions played in the regulation of Vitamin D_3 metabolism was further investigated. Morrissey and Wasserman (156), by studying the effects of differing calcium and phosphate levels, observed that chicks on a high calcium, low phosphate diet, while being hypercalcemic and hypophosphotemic, still were able to have a high intestinal absorption of calcium. Since animals on a low phosphate diet developed a mild hypercalcemia, one would have expected that PTH secretions would have been reduced.

Tanaka and DeLuca (157,158) examined the role played by phosphate and PTH in the regulation of $1,25(\text{OH})_2\text{D}_3$ synthesis. They observed that animals on a low phosphate diet, while being hypercalcemic, were able to synthesize $1,25(\text{OH})_2\text{D}_3$ from intravenously administered $^3\text{H}-25(\text{OH})\text{D}_3$. These observations appeared to be in conflict with the results of Garabedian *et al.* (152), obtained with animals on a low calcium diet. Tanaka and DeLuca were able to demonstrate that animals on a low calcium diet were unable to make $1,25(\text{OH})_2\text{D}_3$ 48 hours after thyro-parathyroidectomy. They also found that if instead of giving PTH to these animals, the animals were fed calcium gluconate and glucose in their water, their plasma phosphate levels

fell to below 8mg/100ml. In this case, without the administration of PTH, these animals were able to synthesize $1,25(\text{OH})_2\text{D}_3$. After three days of this treatment, the animals were able to synthesize $1,25(\text{OH})_2\text{D}_3$ at preoperative levels. Removal of the calcium gluconate-glucose from the water resulted in the animals reverting back to a thyro-parathyroidectomized state in which they produced no $1,25(\text{OH})_2\text{D}_3$. In measuring the plasma phosphate level, it was revealed that as plasma concentration of phosphate decreased to values below 8mg/100ml, $1,25(\text{OH})_2\text{D}_3$ was produced, whereas at values above that figure, $24,25(\text{OH})_2\text{D}_3$ was synthesized. It could therefore be concluded that plasma phosphate concentration in thyro-parathyroidectomized rats was a determinant in $1,25(\text{OH})_2\text{D}_3$ production. No correlation was found between metabolite levels and serum calcium levels. Further studies by these workers (157) demonstrated that there was a strong correlation between renal cortex phosphate content and the ability of these cells to synthesize $1,25(\text{OH})_2\text{D}_3$. It was found that when the phosphate concentration of the renal cortex in these animals was above $400\mu\text{g/g}$ wet weight of tissue, $24,25(\text{OH})_2\text{D}_3$ was synthesized and below that level, $1,25(\text{OH})_2\text{D}_3$ was produced. They reasoned, therefore, that hypophosphatemia which would create a low cellular phosphate concentration resulted in the synthesis of $1,25(\text{OH})_2\text{D}_3$, and the administration of PTH would also lower the phosphate concentration of renal cells, hence promoting the synthesis of $1,25(\text{OH})_2\text{D}_3$. Calcitonin did increase phosphate transfer into renal cortical cells, therefore increasing the phosphate levels. They concluded that the renal phosphate level may, therefore, modulate the synthesis of $1,25(\text{OH})_2\text{D}_3$ which in turn would mobilize calcium from the bone and initiate active transport of calcium from the gut.

Norman and co-workers (159,160) felt that 1-hydroxylase activity, especially in the chick, was regulated by the PTH

directly and by the Vitamin D status of the animal. They were unable to demonstrate any significant relationship between plasma phosphate or renal cortical phosphate concentration and 1-hydroxylase activity in in vitro incubation of chick renal mitochondria. Some of these differences may have been due to species differences, namely in the comparison of chick kidney to rat kidney. These workers had difficulty in demonstrating 24-hydroxylase activity in their incubation system.

4. Physiological and Biochemical Effects of Vitamin D

a. Role of Vitamin D in Calcium Absorption from the Intestine.

Early studies of the intestinal absorption of calcium in the intact animal, as well as in isolated loops of gut, clearly demonstrated that Vitamin D increased the absorption of dietary calcium, especially in Vitamin D deficient rats (148, 161); however, the basic mechanism driving the absorptive process was not understood.

In 1959, Schachter and Rosen (162) successfully adapted the in vitro everted-gut-sac technique of Wilson and Wiseman (163) to study the transport of calcium from the mucosal to the serosal surface. While the in vitro preparation of intestine was not maintained in a true physiological state, the tissue preparation did provide an opportunity to study the transport system under specifically-defined conditions. These workers were able to demonstrate that the intestinal absorption of calcium occurred by active transport, since transport of calcium in vitro occurred against both electrical and chemical gradients.

It was also shown that the intestinal transport system for calcium could move it against a five to ten fold gradient which required oxidative metabolism. Schacter et al. (164) therefore proposed that active intestinal calcium transport involved a two-step process, with net uptake at the mucosal surface, followed by a net transfer toward the serosal surface. Wasserman et al., in 1961 (165) provided similar evidence to support the active transport idea. These findings have

been confirmed by using both in vivo and in vitro techniques (35, 166, 167). More recently, it has been shown (168,169) that the saturation kinetics for calcium uptake at the mucosal border of intestinal epithelial cells was calculated to have a K_m of 1.25 to 2 mM. Furthermore, the translocation process for calcium was independent of phosphate and for optimal activity required Vitamin D (169,170).

Various functional components for the active transport of calcium have been only recently identified. These included a calcium binding protein in the cytosol, a Vitamin D dependent calcium ATPase, mitochondria and a sodium dependent ATPase. In 1966, Wasserman and Taylor were able to demonstrate a Vitamin D dependent calcium binding protein in chick intestinal mucosa (171). This carrier protein was identified in a number of species, including man (172,173). The molecular weight of this carrier protein was between 24,000 and 28,000 (174). Approximately 30% of the amino acid residues were dicarboxylic amino acids (aspartic, glutamic), 10% were leucine, and 8% lysine (174). In rats, the molecular weight of this protein has been found to be 8,000 (175). The calcium binding has been localized near the brush border region and in the goblet cells in the chick duodenum, by means of fluorescent antibody technique (176). A moderate amount has also been demonstrated in the lamina propria region. A high correlation has been shown between binding protein activity and increased calcium absorption following administration of Vitamin D or its active metabolites (156, 177). Also, a higher concentration of calcium binding protein occurred in intestinal mucosa of animals adapted to a low calcium intake (174). The adaption to the low calcium diet and resultant enhancement in calcium transport has been shown to be dependent on protein synthesis, since puromycin has been shown to block increase in calcium binding protein activity and calcium absorption (174). The appearance of calcium binding protein and the increased uptake and translocation

of calcium by mucosal cells did not occur simultaneously, however. Calcium transport appeared to precede protein synthesis (178, 179); however, this may have been due to sensitivity of detection techniques. Moreover, calcium binding protein production continued to increase, even after the rate of calcium absorption had leveled off (178), and rats treated with cortisol maintained a high level of calcium binding protein, while exhibiting depressed rates of intestinal calcium transport (36). However, this may have been due to an effect on the epithelial mitochondria.

Martin et al. (181), in studying the initial uptake of calcium by the mucosal cell, were able to demonstrate that intestinal brush border preparations possessed calcium-dependent ATPase activity that was influenced by Vitamin D (181). They demonstrated that the increase in calcium ATPase activity followed closely the characteristic rise of calcium absorption after Vitamin D was administered to chicks. Further studies in the chick by Haussler et al. (182) elucidated a correlation between both alkaline phosphatase activity and intestinal calcium absorption. They showed that inhibitors of alkaline phosphatase such as alanine, beryllium, L-phenylalanine, theophylline and diphosphonates also inhibited the ATPase activity.

Once the calcium enters the mucosal cell, it is moved to the serosal border. Since the calcium levels could reach a concentration of up to 5 mM, during the absorption, most of the calcium would have to be bound to prevent deleterious effects to the cell (183). It has been observed that intestinal mitochondria from Vitamin D treated animals contain numerous electron-dense granules (184). These granules appeared limited to the microvillus portion in rachitic animals, being sparse in the mitochondria. After Vitamin D administration, the density of the granules in mitochondria increased (184). Mitochondria have been found to accumulate calcium very

rapidly and to maintain a gradient between the cytoplasm in excess of 3000 (185). This process is dependent upon either the oxidation of respiratory substrate or the utilization of ATP to form some high energy intermediate of oxidative phosphorylation (186). Corticosteroids interfere with calcium uptake of mitochondria by interfering with the respiratory chain and in reactions of oxidative phosphorylation (38; 180). Holdsworth (187) demonstrated, by a series of in vitro studies, that the quantity of calcium released from the mitochondria was dependent on the amount of calcium binding protein present in the incubating medium. They suggested that the release of calcium from mitochondria stimulated by the calcium binding protein, aided in the release of calcium from the mitochondria at the serosal cell membrane into the extracellular fluid.

The final step in calcium translocation was shown to involve transporting the calcium out of the epithelial cell and into the extracellular fluid. Martin and DeLuca (188) demonstrated that sodium stimulated the in vitro transport of calcium and suggested that sodium acted on the basal-lateral membrane of the epithelial cell. It has been shown that a sodium-potassium dependent ATPase is located in the serosal membrane (189) and it has been observed that the calcium flux can be inhibited by ouabain when that substance was present in the bath medium on the serosal surface of chick intestinal preparation. It would appear, therefore, that the inward movement of sodium across the serosal boundary into the epithelial cell is coupled with the exit of calcium, hence completing the translocation of calcium across the epithelial cell.

Strong evidence has been presented to show Vitamin D, namely $1,25(\text{OH})_2\text{D}_3$ is responsible for initiating active transport of calcium across the intestinal mucosa. The $1,25(\text{OH})_2\text{D}_3$ was bound to specific cytosol proteins (1,2,3,4,5) before the metabolite was incorporated into nuclear receptors where they affected either the

transcriptional process directly (6, 7) or regulated the release or transport of nuclear-derived RNA. This led to the generation of calcium transport proteins such as the calcium binding protein, alkaline phosphatase and ATPase (8).

b. The Effect of Vitamin D on the Mineralization and Resorption of Bone

i. Mineralization of Bone

As early as 1921, Howland and Kramer (9) demonstrated that the (calcium) X (phosphate) product of blood was low in rickets. Neuman and Neuman (10) emphasized that blood was normally supersaturated with calcium and phosphate when compared with bone mineral. It was later shown (11) that rachitic blood was under-saturated with regard to bone mineral. There, the overall physiological mechanism of Vitamin D was postulated to elevate the plasma calcium and phosphate, which in turn resulted in the laying down of hydroxyapatite in the organic matrix of bone (12, 13). However, there has been recent evidence that Vitamin D or its metabolites may influence the rate of maturation of osteoid and that the cell mediated changes in the bone matrix are involved in mineralization of bone (14-17).

ii. Mobilization of Calcium From Bone

One of the mechanisms postulated to regulate calcium homeostasis was that of mobilization of mineral from bone, due to metabolites of Vitamin D rather than the parent compound. In bone tissue cultures, the $25(\text{OH})_2\text{D}_3$ had calcium mobilizing activity, whereas the parent compound did not (18). $1,25(\text{OH})_2\text{D}_3$ was even more potent than the intermediate metabolite (19, 20). In nephrectomized rats, $1,25(\text{OH})_2\text{D}_3$ was the only metabolite which mobilized calcium when given in physiological doses. The calcium mobilizing activity involved the transcription of DNA and likely protein synthesis (21). Parathyroid hormone augmented the effect of Vitamin D metabolites (22, 23), both in vivo and in vitro. Calcitonin had the opposite effect,

inhibiting calcium mobilization both in the presence and absence of Vitamin D. Apparently these hormones exerted their effects by interacting on different receptor sites on bone cell membrane adenyl cyclase (24). There appeared to be some evidence that the enzyme carbonic anhydrase was involved in bone mineral resorption (25). The authors pointed out that during resorption, the enzyme activity of bone was higher than during accretion. Also the osteoclasts contained carbonic anhydrase while osteoblasts and osteocytes contained no detectable quantities of the enzyme. Furthermore, acetazolamide, a specific inhibitor of carbonic anhydrase, inhibited the hypercalcemic action of estrogen and PTH (25).

c. Renal Effects of Vitamin D

Harrison and Harrison, in 1941 (26), demonstrated a renal tubular effect of Vitamin D, by showing a renal tubular reabsorption of phosphate after Vitamin D administration. However, these studies were carried out in dogs with intact parathyroid glands, therefore it was possible that the effect could have been observed as a result of suppression of PTH secretion. Puschett *et al.*, in 1972 (27), reexamined the action of Vitamin D, $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$ on renal transport of phosphate, sodium and calcium, using thyro-parathyroidectomized dogs. They were able to demonstrate increased phosphate, sodium and calcium reabsorption. Furthermore, they were able to show that $25(\text{OH})\text{D}_3$ opposed the phosphaturic effect of PTH. Brodehl *et al.* (28) were able to demonstrate a similar effect to Vitamin D administration when they used Vitamin D deficient rats.

5. Factors Influencing the Metabolic Fate of Vitamin D

a. Anticonvulsant Drugs

Anticonvulsant drugs such as phenobarbital and diphenylhydantoin are known to cause hepatic microsomal induction (29). Kruse (30) noted an increased incidence of rickets in children who had been administered drugs for several years. Richens and Rowe

(31) demonstrated a direct correlation between total drug dose and the degree of hypocalcemia in epileptic patients. The finding that administration of Vitamin D was effective in treating the rickets (30) and osteomalacia (32) resulted in Hahn et al. (33) examining this phenomenon. They demonstrated an increased rate of metabolism of Vitamin D and $25(\text{OH})\text{D}_3$ to more polar, inactive metabolites. Stamp et al. (34) demonstrated that patients taking antiepileptic drugs had a significantly lower concentration of $25(\text{OH})\text{D}_3$ in their plasma than did control subjects. They further demonstrated that administration of $25(\text{OH})\text{D}_3$ promoted rapid healing of the anticonvulsant induced osteomalacia. These findings clearly showed that while metabolism of Vitamin D was accelerated in patients on antiepileptic medication, the net result was a decrease in the physiologically active circulating metabolite of Vitamin D.

b. Corticosteroids

The antagonistic action of glucocorticoid to calcium absorption is well known (35, 36). Avioli et al. (37) examined the effect of cortisone treatment on the metabolism of ^3H -Vitamin D_3 in normal adult subjects. They observed both a more rapid turnover of Vitamin D and a diminished production of the biologically active metabolite of Vitamin D. Kimberg et al. (36) found little difference in the metabolism of ^3H -Vitamin D_3 in cortisone treated rats; however, they did find that cortisone stimulated the calcium binding protein in the duodenal mucosa. In spite of their contradictory finding, calcium transport was still found to be defective. While this has not yet been resolved, it has been proposed that the glucocorticoids interfere with the sequence of events in the respiratory chain of mitochondria, hence interfering with the calcium transport activity (38).

c. Dietary Strontium

Animals fed a diet containing strontium have been found to develop several imbalances in the calcium homeostatic mechanism, including an inhibition of intestinal calcium absorption, with reduced calcium binding protein (39), as well as the development of rachitic bone lesions. This defect was the result of strontium blocking the synthesis of $1,25(\text{OH})_2\text{D}_3$ by the renal mitochondria, and the synthesis instead of $24,25(\text{OH})_2\text{D}_3$. Administration of $1,25(\text{OH})_2\text{D}_3$ but not $25(\text{OH})\text{D}_3$, restored the calcium absorption in strontium fed chicks (40).

d. Chronic Renal Failure

Chronic renal failure has been shown to be attended by metabolic acidosis, intestinal malabsorption of amino acids (41), calcium (42), an alteration in Vitamin D metabolism and by secondary hyperparathyroidism as well as manifesting itself by defective bone matrix and mineral maturation (42,45). The disordered mineral metabolism in renal failure resulted in the renal osteodystrophy and subsequently, there was a resistance to Vitamin D therapy. The anti-rachitic activity of serum of uremic individuals was much lower than that of healthy control subjects (43). Furthermore, the uremic individuals required larger intakes of Vitamin D for biological effectiveness than did the normal individual (43). The phenomenon of Vitamin D resistance in renal failure led Avioli *et al.* (44) to examine the metabolic fate of labeled Vitamin D in uremic patients. They observed a turnover rate of twice that of normal, in uremic individuals. Furthermore, the anti-rachitic activity of peak IV (containing $25(\text{OH})\text{D}_3$) was about 1/4 that of normal. Follow-up work, using uremic rats (45), demonstrated that the circulating levels of $25(\text{OH})\text{D}_3$ in uremic rats were less than 1/2 that of control animals (3.4 versus 7.5 ng/ml plasma). With the realization of the importance of the role that the kidney played in 'activating' the circulating $25(\text{OH})\text{D}_3$ to its tissue active form, $1,25(\text{OH})_2\text{D}_3$ (60, 190), it has been shown that the loss of renal cells rather than the loss

of kidney excretory function was responsible for the derangement of intestinal calcium transport activity. Therefore, the loss of functional renal cells would result in the reduced production of $1,25(\text{OH})_2\text{D}_3$, and less $1,25(\text{OH})_2\text{D}_3$ would be available for initiating active transport of calcium across the gut. Brumbaugh *et al.* (46) found this to be the case. By means of a competitive protein binding assay, they were able to show that plasma in normal individuals contained an average of 6.4 ng/100 ml of $1,25(\text{OH})_2\text{D}_3$, while plasma from uremic patients contained an average of 2.6 ng/100 ml of $1,25(\text{OH})_2\text{D}_3$. Brickman *et al.* (47) were the first to use $1,25(\text{OH})_2\text{D}_3$ to augment calcium absorption in uremic humans.

The aberration of Vitamin D metabolism may not be the only factor involved in the malabsorption of calcium by the intestinal epithelial cells. There have been reports that uremic toxins, such as indols and guanidines will depress active transport of PAH and uric acid in renal tubules, as well as interfering with the sodium/potassium of red cell ghosts (48). By extrapolation, this effect may also occur in the intestinal mucosa. There has been further evidence that an altered metabolism occurred in intestinal mitochondria (49) which would result in reduced uptake and transport of calcium through the mucosal epithelium.

6. Summary of the Interrelation Between Vitamin D and Calcium Homeostasis

Vitamin D, obtained either from an exogenous source or an endogenous source, has been converted by the liver to $25(\text{OH})\text{D}_3$. The rate of production has been shown to be regulated by a complex substrate product inhibition process. The circulating physiologically active form of Vitamin D, 25-OHD_3 , after being rapidly released by the liver then travels in the blood stream bound to the α -globulin protein. The mitochondria of the renal tubules further hydroxylate the $25(\text{OH})\text{D}_3$ to either $1,25(\text{OH})_2\text{D}_3$ or $24,25(\text{OH})_2\text{D}_3$. The production

of these metabolites is under the servo-control mechanism, involving circulating calcium and inorganic phosphate (Fig. 2). In the event that the concentration of serum calcium and phosphate is somewhat low, this serves as a stimulus to the parathyroid glands to initiate the release of PTH. Low serum phosphate and calcium also inhibit the secretion of calcitonin by the thyroid gland. The net effect of the increase in the PTH plasma levels is that the PTH will act on the renal tubular epithelium to initiate or increase the synthesis of $1,25(\text{OH})_2\text{D}_3$ by the tubular mitochondria. Norman believes that the PTH does this as a direct effect of interacting with the membrane of the renal tubular epithelium. DeLuca has evidence to support the idea that the PTH, by means of its phosphaturic effect on the renal tubules, lowers the intracellular phosphate concentration of the tubular epithelium and this then is the stimulus that initiates the synthesis of $1,25(\text{OH})_2\text{D}_3$. The circulating $1,25(\text{OH})_2\text{D}_3$ then acts on the bowel to initiate active transport of calcium and phosphate, as well as mobilizing both calcium and phosphate from the bone. The resultant elevation of serum calcium and phosphate, the concentration of which is supersaturated, compared to bone, then in turn inhibits the release of PTH by the parathyroid gland, while stimulating the release of increased amounts of calcitonin from the thyroid gland. This is believed by DeLuca's group. Norman recently (94) provided evidence that $1,25(\text{OH})_2\text{D}_3$ may in fact act directly on the parathyroid gland to inhibit the release of PTH. The increase in circulating calcitonin levels acts to stimulate the transfer of phosphate into the renal tubular cell and this resultant elevation of phosphate concentration in the tubular epithelium activates the mitochondrial enzymes responsible for the synthesis of $24,25(\text{OH})_2\text{D}_3$. Thus, according to DeLuca, intratubular phosphate concentration is the major determinant in regulating the synthesis of the two metabolites. Bikle *et al.* (95, 95a) also support the idea of intracellular ionic regulation of the synthesis

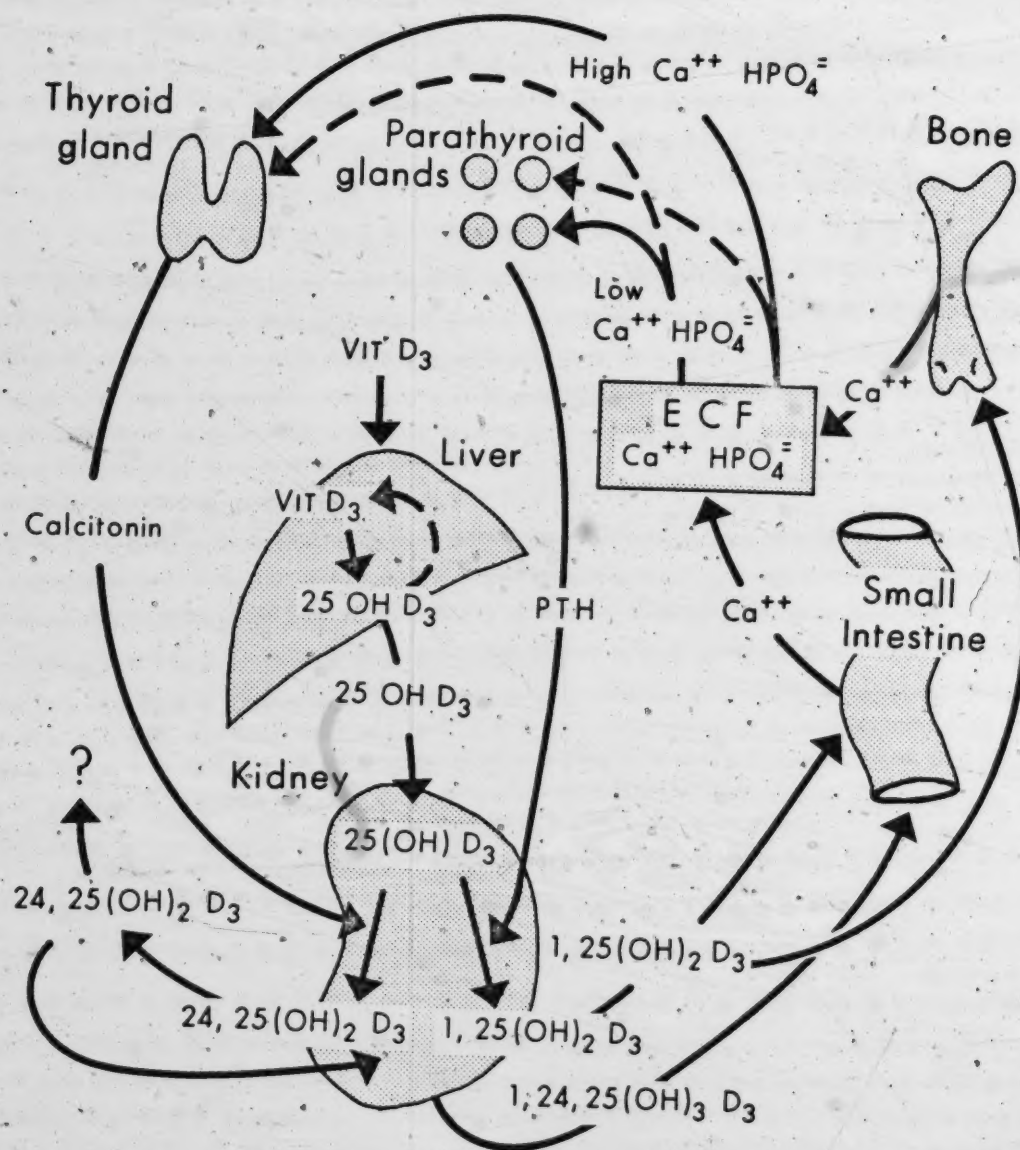


Figure 2. Summary of the Proposed Interrelationship Between Vitamin D and its Metabolites, Parathyroid Hormone, Calcitonin and Calcium Homeostasis.

The dotted lines represent inhibition of either calcitonin or parathyroid hormone released by hypocalcemia and hypophosphatemia and hypercalcemia and hyperphosphatemia, respectively (modified from 219).

of the two metabolites by the renal mitochondria. The exact role of the $24,25(\text{OH})_2\text{D}_3$ is not completely understood, however it is known that the kidney can metabolize the circulating $24,25(\text{OH})_2\text{D}_3$ to $1,24,25(\text{OH})_3\text{D}_3$, which is effective in initiating active transport of calcium in the intestine, however it is inactive in bone.

B. Influence of Uremia on Hepatic Enzymes

Chronic renal failure, with its accompanying metabolic acidosis and accumulating uremic toxins, has been shown to have a definite effect on the various enzyme systems in the body. Mounting, well documented evidence has been accumulated which showed that the uremic pattern altered the normal endogenous pattern of metabolism. It has been shown that the uptake of glucose by tissues (50-54) was altered. Changes in the metabolic pathways have also been noted (52-54).

There is little information available on the lipid metabolism in uremia. Bagdad *et al.* (56) have described a state of hypertriglyceridemia in patients with chronic renal failure as well as many of those being maintained with regular hemodialysis. Fasting triglyceride and immunoreactive insulin levels were elevated which would suggest an increase in hepatic synthesis of triglyceride-rich lipo-protein. Mannan *et al.* (55) also demonstrated an increase in lipid synthesis in chronically uremic rats. They attributed this increase in activity to an increase in utilization of the pentose shunt, or increased hepatic synthesis.

Protein metabolism has also been shown to be altered significantly in uremia. McCormich *et al.* (57) demonstrated that amino acids in uremic liver homogenates were more readily incorporated into liver protein. These findings were supported by Shear (58) in their liver function studies. It was also observed that the perfused uremic livers produced more urea than control liver. Work by Hoppe-Seyler *et al.* (59)

revealed that the activities of the two rate limiting enzymes in the urea cycle are altered in the uremic state.

There is a significant quantity of evidence which has suggested that many enzymes are actually inhibited by the uremic toxin per se (72) (urea, methyl guanidine, guanidino-succinic acid). It has been shown by Glaze et al. (71) that human uremic serum ultrafiltrate uncouples oxidative phosphorylation at the phosphorylation site linked to the respiratory chain between NADH and cytochrome b in rat liver mitochondria. It has also been shown that uremic serum will cause acceleration of glucose oxidation by rat liver (73) and inhibit lactic dehydrogenase activity (74).

Aside from the endogenous enzyme systems in uremic patients and experimental animals, there have been few published studies on the effect of uremia on the metabolism of exogenously administered compounds. Most foreign compounds, including therapeutic drugs, are metabolized by the liver and then excreted in a free metabolic or conjugated form in the bile or urine. The therapeutic effectiveness and toxicity of a drug has been found to be a function of its rate of biotransformation; only a limited number of drugs that are metabolized have been studied in the uremic syndrome in such a manner that information could be gained about drug metabolism in uremia. It has been shown that some of these drugs have normal half-lives while others seem to be prolonged, while still others appear to have a shortened half-life. Drugs may be metabolized by oxidation, reduction and hydrolysis. Furthermore, they may then be conjugated to polar molecules such as glucuronide acid or glycine. The resulting product would be much more polar than the parent compound and could therefore be easily excreted in the bile or urine.

An excellent example of drug oxidation is the metabolism of Vitamin D₃. It has perhaps been the most thoroughly investigated therapeutic agent in uremia. Vitamin D has to be hydroxylated at the

aliphatic 25-C position to become the circulating active form of the vitamin (13). Avioli *et al.* (44) demonstrated that in uremic patients, while there was an accelerated general hydroxylation of calciferol, there was a very significant reduction of the 25-hydroxylation reaction.

Glogner *et al.* (61) studied tolbutamide oxidation in uremic patients. They found that the seven uremic individuals had normal tolbutamide half-lives. However, the half-life of the biologically inactive carboxy tolbutamide that was eliminated by means of urinary excretion, was markedly prolonged.

Phenobarbitol, eliminated mainly by oxidation, has been shown to be metabolized similarly in both uremic and normal individuals (62). In contrast to these findings, it was observed that the sleeping time for the short and intermediate acting barbiturates, eg., thiopental, hexabarbitol, and pentabarbitol, was prolonged in uremic animals (63, 64).

Diphenylhydantoin is oxidized to 5-parahydroxydiphenylhydantoin which is then conjugated with glucuronic acid and then excreted in the urine. Letteri *et al.* (65) showed that the plasma half-life of the drug was shorter in uremics than in normal control individuals.

The metabolism of aminopyrine was studied in uremic and normal subjects (66). Maddocks *et al.* (66) observed that the plasma half-life was considerably shortened in uremic individuals.

Conjugation of other drugs and their metabolites is also influenced by uremia. Acetylation of drugs, particularly the sulfonamides, (sulphadimidine and sulphamethaxole) was significantly increased in the majority of patients studied (67, 68), taking into consideration the fact that there are genetically controlled rates of acetylation. Reidenberg *et al.* (69) demonstrated contrary evidence, indicating that sulphisoxazole acetylation was slowed. All three studies indicated that there was a delay in excretion of the conjugated drug

from the plasma in uremia. Conjugation of chloramphenicol with glucuronide acid has been shown to be unchanged in uremics (70).

It would appear, from these studies, that the data for many of these pathways are inconclusive at best.

C. The Effect of Stress, Specifically Cortisone Levels on Drug Metabolizing Enzymes.

The responses to stress, with its activation of the pituitary-adrenal axis, and adrenal hypertrophy have been well documented (75). Bousquet and co-workers (76-78), in a series of elegant experiments, were able to demonstrate the role of the pituitary-adrenal axis in the response of accelerated metabolism to the stimulus of stress. They were able to demonstrate that subjecting rats to the stress of hind limb ligation for two and one half hours, shortened the duration of pharmacological response to hexabarbital, pentobarbital, meprobamate and zoxazolamine; that is, the plasma levels of these agents were reduced significantly, compared to the controls. These workers were also able to demonstrate that metabolism increased in perfused rat liver obtained from animals treated similarly (77,78). The role of the pituitary-adrenal axis in stimulating drug metabolism was demonstrated in hypophysectomized or adrenalectomized animals. These animals were unable to demonstrate an increase in metabolism. Their response could be reversed when corticosterone, but not ACTH, was administered to adrenalectomized animals. In hypophysectomized animals, both cortisone and ACTH administration increased drug metabolism. Treatment of rats with actinomycin D blocked the ability of stress to lower the pentobarbital levels in both in vivo and in vitro (perfused livers), suggesting that increased protein, hence enzyme, synthesis may have been involved in the stress-mediated enzyme induction.

The role of the adrenal cortex and steroid hormones on drug metabolizing enzymes was illucidated by Cox and Mathias (80). They were able to demonstrate the induction, by cortisol, of tryptophan pyrrolase in the liver. They were also able to demonstrate that cortisol administration to control and adrenalectomized animals stimulated both free and bound ribosomes and polyribosomes to incorporate amino acids into protein, thus stimulating protein synthesis in the liver.

Castro *et al.* (81) demonstrated that the stimulating effect of cortisol in adrenalectomized rats was not great on the cytochrome P-450, but that it had a very significant effect by increasing the levels of NADPH cytochrome c reductase and cytochrome P-450 reductase activity. They came to this conclusion by observing that the N-demethylase activity (substrate being ethylmorphine) paralleled that of the reductase enzymes and not that of the cytochrome P-450.

It has been demonstrated that steroid hydroxylase and drug oxidase systems in the liver are very similar (82, 83). Other workers (84), including Tephyl and Mannering, pointed out that both the administration of steroid hormones *in vivo* (84) and *in vitro* (85) could competitively inhibit the oxidation of drugs, which supported the theory or concept that drugs and steroids are alternative substrates to oxidative enzymes.

D. Uremic Models: Different Methods used for the Induction of Experimental Uremia.

In order to study some of the various metabolic dyscrasias which occur in uremia, without using human subjects, researchers have developed a number of uremic animal models to meet their requirements.

Bricker *et al.* (86, 87) described a method of inducing chronic uremia in the dog and rat. The left kidney was exposed, and punctured 50 times with a 26 gauge cautery needle. An additional 50 punctures were then made with a 25 gauge needle that had been dipped repeatedly

into a culture of Escherichia coli. Partial infarction was then induced by tying the majority of the primary branches of the renal artery. Contralateral nephrectomy was performed a week later.

A number of workers including Janike (88) have used intravenous hemaglobin administration in dogs and rats. It was felt that the most likely cause of renal failure was due to obstruction in the proximal convoluted tubules caused by hemoglobin casts. Others (89) have used intramuscular or intraperitoneal injections of glycerol to induce renal failure. The primary lesion was found to be extensive necrosis of proximal tubular epithelium. Shafrir et al. (90) described a procedure whereby they injected an aminonucleoside of puromycin (6-dimethyl-amino-9-[3'-(p-methoxy-1 phenylalanylmino-3'-deoxy- β ,D ribofuranosyl] -purine), subcutaneously seven to nine times daily.

Schweibe et al. (91) described a method of occlusion of the renal vein for the various periods of time, followed by contralateral nephrectomy a week later. Avioli et al. (92) described a modification of the method of Bricker (86) whereby they selectively ligated the main branch arteries which leave the renal artery to supply the upper and lower poles of the kidney. This resulted in an infarction to 25 to 85 percent of the kidney. Contralateral nephrectomy was performed one week later.

A final method was the one used by Kessner and Epstein (93). This procedure involved removing the upper and lower poles of the left kidney, using a midline abdominal approach, so that 60 to 80 percent of the functional renal mass was removed. Gelfoam[®] or Surgicel[®] was then sutured in place over the exposed portions of the renal surface. Prior to this the adrenal gland was freed and care was taken to ensure that it was not removed during surgery. A week later the contralateral kidney was removed by means of a flank approach.

III. EXPERIMENTAL METHODS AND MATERIALS

A. Chemicals and Reagents

All chemicals and solvents used were of analytical reagent grade. Nutritionals such as glucose-6-phosphate, nicotinamide adenine dinucleotide phosphate (NADP), and adenosine triphosphate nucleotide (ATP) were obtained from Nutritional Biochemicals (Cleveland, Ohio), glucose-6-phosphate dehydrogenase was obtained from Sigma Chemical (St. Louis, Missouri). Crystalline bovine serum albumin was purchased from Mann Research Laboratories (New York, U. S. A.). Solutions of the various substrates and cofactors were prepared with either distilled water or 0.1 M phosphate buffer. Concentrations of various cofactors and substrates used are described in the text.

^{14}C -4-Vitamin D_3 (32.5 mCi/-mM) was obtained from Amersham-Searle (Toronto, Canada). Upon receipt the material was diluted with 1 ml benzene-absolute ethanol (1-9 v-v) and divided into five 0.2 ml aliquots. These were in sealed ampules under an atmosphere of nitrogen and were stored at -18°C . When required for experimental use the solvent of the isotope solution was removed by a stream of nitrogen gas and the labeled material was then redissolved in absolute 'super dry' ethanol. Radiochemical purity was measured by thin layer and column chromatography. Radiochemical purity of better than 95% was confirmed. The labeled Vitamin D_3 was also compared to a reference compound of unlabeled Vitamin D_3 (Eastman Kodak) by co-chromatography and absorption spectrophotometry.

^3H -(26,27)-25-hydroxy-Vitamin D_3 (6-20.0 Ci/mM) was treated as described for Vitamin D. Examination for radiochemical purity by thin layer and column chromatography demonstrated a radiochemical purity of better than 98%. Co-chromatography with authentic $25(\text{OH})\text{D}_3$, obtained as a gift from the Upjohn Company of Kalamazoo, Michigan, further confirmed the identity of the compound.

B. Animals and Diet

Male Wistar rats, obtained from Woodlyn Farms Ltd. (Guelph, Ontario) weighed 175-200 g at the onset of the experiments. The animals were individually housed in hanging wire cages, and kept in a room at constant temperature (22°) with twelve hours of light per day. They were allowed food and water ad. lib. Control and uremic rats were maintained on Purina Rat Chow. Animals which were kept on a rachetogenic diet were fed a pelleted form of the Rachetogenic Diet No. 2 (Nutritional Biochemicals - Cleveland, Ohio).

The chicks used in the experiments were 'Rapid Gro' broilers, obtained from Dominion Hatcheries (Edmonton, Alberta); and were maintained on 'Chick Starter' (Northwest Feed Co., Edmonton, Alberta).

1 C. Preparation of the Animal Model

1. Surgical Approaches Used

a. Renal Occlusion Technique (91)

Three groups of eight rats each, were subjected to occlusion of the renal vein for periods of 30, 45, and 60 minutes respectively. Seven days later the contralateral kidney was removed by means of a flank incision. The blood urea nitrogen (BUN), weight gain, and water consumption were monitored.

b. Selective Renal Artery Ligation Technique (92)

Eight rats were subjected to selective renal artery ligation which resulted in infarction of 25 to 85% of the renal mass. One week later the contralateral kidney was removed.

c. Partial or Subtotal Nephrectomy (93)

Surgical removal of 60 to 80% of the total renal mass was achieved in a two-stage operation. The first stage involved the removal of about two thirds of the left renal mass; the upper and lower poles of the kidney were removed after the vessels had first been clamped. The exposed portion of the remaining renal mass was

covered by Gelfoam[®] (Upjohn, Kalamazoo, Michigan) or 'Surgicel'[®] (Johnson & Johnson, New Brunswick, New Jersey) and sutured into place prior to the release of the clamp. During the removal of the upper pole of the kidney, care was taken to ensure that the adrenal gland was left intact.

An alternate method used was that of ligating the upper and lower poles of the left kidney so that less than one third of the kidney had a viable blood supply. A week later the contralateral kidney was removed. Again weight gain and BUN was monitored.

2. Parameters Used for Evaluation of the Uremic Model

Blood urea nitrogen (BUN) was one of the main parameters used to measure the effectiveness of the experimental procedure used to induce uremia. The blood levels were monitored every two weeks in both experimental and control animals. BUN determinations were performed in the laboratories of the Surgical Medical Institute (Edmonton, Alberta) according to the method of Chancy and Marbach (191). Other determinations were made in our laboratory using 'Eskalab'[®] (Smith Kline Laboratories, Palo Alto, California) reagent tablets for the determination of BUN.

Body weights were recorded weekly in both experimental and control animals, water consumption was also noted. At the termination of the experiment the femurs were X-rayed to get an indication of radiodensity. Various soft tissues, especially the liver and kidneys, were also examined for histopathological changes.

D. Procedures Used for Determination of In Vivo Metabolism of ^{14}C -4-Vitamin D_3 and ^3H -(26,27)-25-OH-Vitamin D_3

1. Administration of Labeled Compounds

The labeled compounds, Vitamin D_3 and 25(OH) D_3 were administered intraperitoneally in the initial studies and intra-

venously in later studies; the vehicle for the vitamin used was 95% ethanol given intraperitoneally in volumes of 100 μ l, and intravenously 50 μ l.

In the first series of experiments, control, uremic and unilaterally nephrectomized rats were given 100 μ l of ethanolic ^{14}C -Vit D₃ (0.5 μ Ci, 240 I. U.) intraperitoneally. Serial blood samples were taken from the ventral coccygeal vein in 100 μ l aliquotes over a 24 hour period. After this period of time the animals were exsanguinated while under light ether anesthesia. The whole blood was centrifuged and the plasma was collected and stored in a frozen state (-10⁰) for future chromatographic analysis. Other soft tissues such as liver, kidney and small intestine were also removed and stored in a frozen state.

In order to determine the total activity of the whole blood, representing vitamin D and its metabolites, the samples were treated essentially as described by Wiebe *et al.* (192). The aliquots of blood were placed into a liquid scintillation vial and 2.5 ml of hydrogen peroxide were added very slowly. The sample was ultrasonicated with an ultrasonic probe, after which 10 ml Aquasol[®] (New England Nuclear) was added and the sample was again treated with the probe. Following this 2 to 3 drops of stannous chloride solution was added. The samples were then placed into a refrigerated Liquimat 220 (Picker Nuclear, White Plains, New York) liquid scintillation spectrometer and were dark adapted for 2 hours before scintillation counting. Quench correction was achieved by means of the External Standard Ratio technique.

2. Extraction of Vitamin D₃ and its Metabolites

The frozen samples were thawed and extracted by means of a procedure similar to that described by Blight and Dyer (109). To the plasma samples 1 volume of chloroform and two volumes of methanol were added. The samples were placed into glass-stoppered conical vials and were shaken mechanically for 30 minutes. They were then allowed to stand overnight in a cold room. The following day one volume of distilled water and one volume of chloroform were

added in addition to approximately 0.5 g $(\text{NH}_4)_2\text{SO}_4$. The samples were then shaken, centrifuged for 15 minutes at 2000 rpm, the chloroform phase was removed, and the sample reextracted with a similar volume of chloroform. The two extracts were then combined in a 50 ml round bottomed flask and the chloroform was removed from the lipid extract by means of a flash evaporator. The residue was redissolved in one ml of chloroform-hexane (65-35 v-v) and placed on a Sephadex LH-20[®] (Pharmacia Fine Chemicals Inc., Piscataway, New Jersey) column for chromatographic analysis.

Other tissues were treated similarly, with the exception that the tissues and the chloroform-methanol were blended in a Virtis (Gardiner, New York) homogenizer.

3. Chromatographic Analysis

The various lipid soluble tissue extracts were chromatographed by means of column chromatography. The method used was similar to the one described by Holick and DeLuca (112). Twenty-five grams of Sephadex LH-20 was slurried in 100 ml of chloroform-hexane (65:35, v:v). After 24 hours equilibration the slurry was poured into a 50x1.3 cm water jacketed glass column containing 15 to 20 ml of similar solvent. The stopcock was opened as the slurry was poured. The column was allowed to settle by gravity and a further 200 ml of the solvent was passed through the column to aid in packing the bed. The lipid extract was placed on the column and constant volume fractions were collected by means of a Buchler (Buchler Instruments, New York) fraction collector. The fractions were transferred from the collection tubes to liquid scintillation vials, the collecting tubes were rinsed once with three ml chloroform to ensure quantitative transfer of the fraction. The wash was added to the vials. The vials were placed in a water bath and the solvent was evaporated. Finally, 10 ml of toluene based fluor (4g PPO, 50 mg POPOP in 1 liter toluene) was added to each vial and the radioactivity was determined by means of a liquid scintillation

spectrometer. Quench correction was achieved by means of the external standard technique using appropriate standards.

E. Determination of In Vitro Metabolism of Labeled Vitamin D₃ and 25-hydroxy-Vitamin D₃

1. Tissue Preparation

The animals were placed under light ether anesthesia and exsanguinated by means of abdominal aortic puncture. The liver and/or kidneys were rapidly removed and placed in an ice cold 1.15% KCl or 0.25 M sucrose solutions. They were rinsed and freed of loose connective tissue and then weighed.

2. Assay Procedures

a. Preparation of Liver Homogenates

Several different methods were employed in order to study the in vitro metabolism of ¹⁴C-4-Vitamin D₃ by uremic and control liver homogenates.

In the initial trials of in vitro studies the liver was homogenized in 4 volumes of 1.15% ice cold KCl by means of a Virtis blender. Both homogenates and 9000xg supernatants were tested. The 9000xg supernatant was prepared by centrifuging the liver homogenate in a refrigerated Sorvall RC-2B medium speed centrifuge using a SS-34 type rotor.

To a series of 50 ml Erlenmeyer flasks the following cofactor solutions were added: 0.5 ml NADP (2 μmoles), 0.5 ml glucose-6-phosphate (25 μmoles), 0.25 ml MgCl₂ (25 μmoles), 0.25 ml nicotinamide (100 μmoles), and 2.5 ml 0.1 M phosphate buffer pH 7.4 (250 μmoles). Ten microlitres of ethanolic ¹⁴C-Vitamin D₃ (0.2 μCi) was added to the cofactors. Then 2 ml of the homogenate or the 9000 xg supernatant was added to the incubation mixture. Incubation was carried out in a Dubnoff Incubator at 37°C for 2 hours under air. Fifteen ml of chloroform:methanol (1:2) was

added to stop the reaction. The monophasic mixture was allowed to stand overnight in the cold room. The lipid soluble material including Vitamin D and its metabolites was extracted according to the method of Blight and Dyer (109) as previously described. The extract was then prepared for Sephadex LH-20 chromatography.

Since there was a suggestion that the oxidation of Vitamin D may be of mitochondrial origin (Horsting and DeLuca, 124), sodium malate was included in the incubation mixture. It has been shown that malate or succinate form an integral part in the mitochondrial oxidative electron transport chain (Estabrook 193). Therefore a series of experiments were performed to assess the effect of malate. The procedure followed was similar to the one described above, however 0.5 ml malate (50 μ moles) in 0.1 M phosphate was added.

The third series of experiments were performed using a threefold quantity of liver homogenate in order to increase the enzyme content of the incubation mixture while using the same quantity of substrate. To a 100 ml Erlenmeyer flask was added 3 ml NADP (12 μ moles), 3 ml glucose-6-phosphate (150 μ moles), 1.5 ml $MgCl_2$ (150 μ moles), 1.5 ml nicotinamide (600 μ moles) and 2 ml Na malate (200 μ moles). Instead of using the Virtis blender, a Potter-Elvehjem homogenizer with a teflon pestle was used. The incubation conditions were as previously described as were the methods of extraction.

The fourth system used was that described by Bhattacharyya and DeLuca (125) to measure rat liver calciferol-25-hydroxylase. The livers were placed in ice cold 0.25 M sucrose, blotted dry, weighed and then minced. The portions of liver were then homogenized in 3 volumes of 0.25 M sucrose using a Potter-Elvehjem homogenizer with a teflon pestle. To 5 ml homogenate was added 10 μ l of 95% ethanolic ^{14}C -Vitamin D₃ (0.2 μ Ci). The mixture was

blended using three strokes with the Potter homogenizer and was then transferred to a 125 ml Erlenmeyer flask. The homogenizing vessel was rinsed with a 2.5 ml 0.1 M phosphate buffer-cofactor solution containing 1 μ mole NADP, 400 μ moles nicotinamide, 50 μ moles ATP, 56 μ moles glucose, and this was then transferred to the homogenate. Subsequently 2.5 ml of salt solution of 5 mM MgCl_2 , 0.1 M KCl was added and the flasks were placed in a metabolic incubator (Dubnoff) and incubated at 37°C for 2 and 4 hours. The reaction was stopped by the addition of 30 ml of chloroform - methanol (1:2) and extracted as previously described.

b. Preparation of Kidney Homogenates for 25-OH Vitamin D-hydroxylase Activity.

Several different procedures were used for the in vitro assay of 25-hydroxy-Vitamin D hydroxylase activity in rat renal mitochondria.

The first method used was similar to that described by Boyle et al. (140). A 20% homogenate of rat kidney was prepared by adding 4 volumes of 50 mM Tris HCl buffer in 0.2 M sucrose to minced kidneys which were then homogenized by a Potter homogenizer. To a 125 ml Erlenmeyer flask the following cofactor solutions were added: 4 μ moles NADP, 40 μ moles glucose-6-phosphate, 180 μ moles nicotinamide, 20 μ moles MgCl_2 , and 100 μ moles Na malate for a total volume of 4 ml. To this, 6 ml of the 20% renal homogenate was added, as well as 0.2 ml glucose-6-phosphate dehydrogenase (10 units) and 20 μ l of ethanolic ^3H -(26,27)-25-OH Vitamin D_3 (0.4 μ Ci). The flasks were flushed with oxygen for two minutes, stoppered, and incubated at 37°C with continuous gentle agitation (90 oscillations per minute) in a Dubnoff Incubator. At the end of 2 hours the reaction was stopped by the addition of 30 ml chloroform-methanol (1:2, v:v) and extracted as previously described. The lipid soluble extract was then chromatographed using either Sephadex

LH-20 chromatography (Holick and DeLuca, 112) or by means of thin layer chromatography (151, 194).

Due to inconsistent results, the in vitro technique was tested using young chick renal homogenates. A 25% homogenate was prepared using 3 volumes of 20 mM TrisAcetate-0.25 M sucrose (pH 7.4) solution. To a 50 ml Erlenmeyer flask was added 0.25 ml NADP (1 μ mole), 0.25 MgCl_2 (5 μ mole), and 0.25 ml Na succinate (1.0 μ moles). Then 2 ml of chick kidney homogenate was added as well as 10 μ l ethanolic ^3H -25(OH) D_3 . The incubation took place at 38° under an atmosphere of pure oxygen (flow rate - 1800 ml per minute). The duration of incubation was one hour. The reaction was stopped with 15 ml chloroform-methanol (1:2, v:v) and extracted as previously described.

This method was repeated in both control and uremic rat kidney homogenates. The only variation was using both 1 and 2 μ moles of NADP in the incubation mixture.

3. Chromatographic Analysis

The above extracts were examined by means of column chromatography and thin layer chromatography.

a. Column Chromatography

The chloroform extract was concentrated by means of a flash evaporator and then redissolved in chloroform-hexane (65:35, v:v). All lipid extracts were applied to water-jacketed glass columns, 1.3x50 cm containing 25 g of Sephadex-LH-20 equilibrated with 65% chloroform in hexane (112). To aid in better resolution between Vitamin D_3 and 25(OH) D_3 a less polar system, namely 50% chloroform in hexane, was used. Fractions were collected in a fixed volume Buchler fraction collector. The radioactivity of each fraction was determined as previously described. A graph of activity versus volume was plotted to determine the presence of the various metabolites. The location of the Vitamin D_3 , 25(OH) D_3 and 1,25(OH) $_2\text{D}_3$ was determined by the use of authentic reference compounds. Their elution volumes compared to published values (112).

b. Thin Layer Chromatography

Thin layer chromatography was considered since it is more rapid when being used as a screening technique. Two types of solvent systems were used. The first was 100% ethyl acetate (151), the second, ethyl acetate:hexane (1:1 v:v) (194). Chrom-AR 1000[®] Mallinkrodt strips 2.5 cm by 20 cm were used. The strips were first activated by being heated in an oven at 120°C. After being cooled to room temperature, 25 μ l of the chloroform extract was spotted on the origin. The spot was then dried by blowing N₂ gas over it. The strip was placed into a sealed 2 litre dark brown glass jar and the strip was allowed to equilibrate with the solvent for about 1/2 hour. The strip was then lowered into the solvent and was removed when the solvent front had traveled 17 cm. It was cut into 1 cm strips which were placed into liquid scintillation vials, 10 ml of fluor was added and this was counted for activity. A more reliable alternate method was also used. This involved leaching the activity out of the Chrom-AR by adding 2 ml each of diethyl ether, chloroform and ethanol and allowing it to stand for several hours. The Chrom-AR strip was then removed. Most of the activity was extracted in this manner. Reextraction of the strips demonstrated very little activity. The solvents were then evaporated, by means of a hot water bath in a fume hood. Ten ml of LS fluor (4 gm PPO., 50 mg POPOP in 1 l toluene) was added and the samples counted for 10 min in either a Liquimat 220 or a Searle Mark III (Des Plaines, Illinois) Liquid Scintillation Spectrometer.

F. . Determination of Hepatic Microsomal Enzyme Activity

The enzyme assays used in these experiments to assess the effect that uremia has on microsomal oxidative enzymes were, essentially, standard published methods. They were modified, as required, for

this series of experiments. To compensate for circadian rhythm of microsomal enzymes, animals were sacrificed between 8 a. m. and 10 a. m.

Tissue Preparation

Each of the rats was exsanguinated after having been placed under light ether anesthesia. The entire liver was removed and placed in ice cold isotonic 0.25 M sucrose solution. The whole livers were weighed, and divided into smaller portions which were again weighed. These portions were minced and homogenized in 3 volumes of 0.25 M sucrose, using a Potter-Elvehjem homogenizer with a teflon pestle. The homogenate was centrifuged at $15,000 \times g$ for 20 min at $2-4^{\circ}\text{C}$ in a Sorvall refrigerated centrifuge, using a fixed angle SS-34 rotor. The supernatant was transferred to chilled clean glass test tubes, with care being taken so as not to include the lipid layer on the surface of the contents of the centrifuge tubes. The $15,000 \times g$ supernatant was gently mixed to ensure homogeneity throughout the sample. Aliquots of the supernatant were then used to assay for specific enzyme activity, as well as for cytochrome P-450 and microsomal protein.

1. Determination of Hexobarbital Oxidase

For the assay of hexobarbital oxidase, the following solutions were added to a 25 ml Erlenmeyer flask: 0.4 ml sodium hexobarbital (2 μmoles), 0.25 ml NADP (1 μmole), 0.5 ml glucose-6-phosphate (25 μmoles), 0.2 ml nicotinamide (20 μmoles), 0.25 ml MgCl_2 (25 μmoles), 1 ml $15,000 \times g$ supernatant and 0.1 M phosphate buffer pH 7.4, to make 4.5 ml. Incubation blanks were prepared in a similar manner, without the addition of the $15,000 \times g$ supernatant. The substrate was added in both the above quantity and double the above quantity. Incubation was carried out in a Dubnoff metabolic incubator, at 37°C under air. Speed of the shaker was adjusted to about 90 oscillations per minute. The assay procedure for the measurement of hexabarbital metabolized was essentially that described by Cooper and Brodie (195). After 1 hr of incubation, the reaction mixture was arrested by the addition of 2 ml (0.1 M) phosphate buffer pH 5.5.

followed by 1 g NaCl. The contents of the incubation mixture were transferred to 45 ml stoppered test tubes. To this was added 30 ml heptane, containing 1.5% isoamyl alcohol. The large stoppered test tubes were placed in a mechanical shaker, to be shaken for 45 min. The test tubes were then centrifuged in a low speed International Equipment centrifuge, at 2000 rpm, to aid in the break-up of any emulsion that may have formed. Twenty ml of the solvent phase was then placed into clean 45 ml stoppered test tubes, to which was added 4 ml of 0.8 M phosphate buffer pH 11. The test tubes were stoppered and shaken for 5 min in a mechanical shaker. The samples were then centrifuged to remove the solvent from the aqueous phase. Three ml of the aqueous phase was placed in quartz cuvettes and the optical density at 245 m μ was determined by means of a Unicam P 1800 spectrophotometer. As a blank, basic phosphate buffer, shaken with the solvent and centrifuged, was used. A standard curve was prepared by adding known quantities of aqueous hexobarbital to a denatured incubation mixture. The extraction procedure was followed as described. A standard curve was prepared, to be used for the determination of the un-metabolized hexobarbital. Extraction was almost complete, i. e. more than 99% (actually 66%) of the hexobarbital could be recovered. This apparent low value represents 20 ml of heptane solvent used, of the total 30 ml used for the initial extraction. The assays were always carried out in duplicate.

2. Determination of Aminopyrine Demethylase

The method used here was essentially that of Gram *et al.* (196) 1968 (and LaDu *et al.*, 197). The reaction mixture for this assay contained 1.0 ml aminopyrine (10 μ mole), 0.25 ml NADP (1 μ mole), 0.25 ml glucose-6-phosphate (12 μ moles), 0.25 ml MgCl₂ (25 μ moles), 0.25 ml nicotinamide (100 μ moles), 0.5 ml semicarbazide (50 μ moles), 3.0 ml 0.1 M phosphate buffer pH 7.4. To this was added 0.5 ml 15,000 x g supernatant, to make up a total volume of 6 ml in a 25 ml Erlenmeyer flask. The sample flasks were incubated aerobically for 30 min at

37°C in a Dubnoff Metabolic Incubator, as previously described. The amount of formaldehyde formed from the substrate was determined by the method of Nash (198) as modified by Cochin and Axelrod (199). The incubation reaction was terminated by the addition of 2 ml of 20% ZnSO_4 , followed after 5 minutes by the addition of 2 ml saturated barium hydroxide solution. The contents were transferred to centrifuge tubes and were centrifuged at 2000 rpm for 15 min. Five ml aliquots of protein free supernatant were treated with 2 ml of double strength Nash reagent (75 g ammonium acetate, 1 ml acetylacetone and distilled water to a final volume of 250 ml). The reaction mixture was treated in a water bath at 60°C for 30 minutes to allow for color development. The solutions were then cooled and optical density, at 415 μm , was determined by means of a Unicam P 1800 spectrophotometer. The quantity of formaldehyde formed was determined from a standard curve which was prepared by adding known quantities of formaldehyde to incubation flasks and performing the assay procedures, as described.

3. Determination of Para-nitrobenzoic Acid Reductase

The incubation mixture for the determination of rat liver microsomal para-nitrobenzoic acid reductase activity was as follows: to 20 ml beakers were added 2.0 ml para-nitrobenzoic acid (6 μmoles), 0.5 ml NADP (2 μmoles), 0.5 ml glucose-6-phosphate (25 μmoles), 0.25 ml MgCl_2 (25 μmoles), 0.25 ml nicotinamide (25 μmoles) and 1.5 ml 0.1% phosphate buffer pH 7.4, for a total volume of 5 ml. The beakers containing the substrate and the cofactors were then placed in a Dubnoff Metabolic Incubator and a lucite chamber was placed over the beakers. While the mixture was being incubated at 37°C, the lucite chamber was flushed with nitrogen gas for 15 min, using a flow rate of about 1500 ml/min. Then 1 ml 15,000 x g supernatant was added to each beaker with a hypodermic syringe, through small holes above each beaker, therefore allowing the nitrogen atmosphere to remain intact. The reaction mixture was then incubated

at 37°C for 30 min and then arrested by the addition of 4 ml of 20% trichloroacetic acid. The mixture was transferred to test tubes and centrifuged for 15 min at 2000 rpm. A 4 ml aliquot of the protein-free supernatant was used to measure the free para-nitrobenzoic acid which was formed. Another 4 ml aliquot of supernatant was hydrolyzed by the addition of 0.3 ml of concentrated HCl and was heated on a boiling water bath for 30 min. The latter aliquot was used for the determination of the total, (i.e. free plus conjugated) quantity of metabolite. The quantitation of metabolite was achieved by the method of Bratton and Marshall (200). To each of the 4 ml aliquots was added (at approximately 10 min intervals) the following: 1 ml of 0.1% sodium nitrite, 1 ml of 0.5% ammonium sulphamate and 1 ml of 0.1% N-(1-naphthyl)-ethylenediamine HCl. After 20 min, the optical density of the pink solution was measured at 540 mμ, using a Unicam P 1800 spectrophotometer. The quantities of para-aminobenzoic acid formed were calculated from a standard curve which was prepared by adding known quantities of para-aminobenzoic acid to 1 ml of 15,000 x g supernatant and cofactors and recovering the para-aminobenzoic acid according to the assay procedure as described.

4. Determination of Microsomal Cytochrome P-450

The cytochrome P-450 content was determined by measuring the dithionite reduced plus CO minus dithionite reduced, difference spectra of liver microsomes, from 450 to 490 mμ, essentially as described by Omura and Sato (201). Two ml aliquots of the 15,000 x g supernatant were placed into polyethylene ultracentrifuge tubes and to these, 8 ml of ice cold 1.15% KCl was added. The samples were then placed into a Beckman Type 40 fixed angle titanium rotor and centrifuged at 105,000 x g for 60 min, in a Beckman L550 preparative ultra-centrifuge. The soluble fraction (105,000 x g supernatant) was decanted and 6 ml of ice cold 0.05 M phosphate buffer pH 7.4

containing 10^{-3} M disodium EDTA was added to the pellet. The pellet was broken up by means of a small teflon pestle and was then resuspended with the aid of a pasteur pipette. The tubes were kept cool in an ice bath. The microsomal suspension was then divided between two matched cuvettes fitted with ground glass stoppers. A baseline was recorded, using a Unicam P 1800 spectrophotometer, by scanning from 400 to 500 m μ . Carbon monoxide was then gently bubbled through the sample cuvette for a period of 20 to 30 seconds. A few mg of crystalline sodium dithionite was added to both sample and reference cuvette. The cuvettes were inverted 10 times. The sample cuvette was then gassed again with carbon monoxide for a further minute. The difference spectrum was then recorded from 400 to 500 m μ . The difference in optical density was determined as a change between 450-490 m μ and the quantity of cytochrome P450 was determined using the molar extinction coefficient of $91 \text{ mM}^{-1} \text{ cm}^{-1}$ (201).

5. Microsomal Protein Determination

The microsomal protein content of the $15,000 \times g$ supernatant was determined by a method based upon the colorimetric determination of Lowry *et al.* (202), as modified by Miller (203). To 1 ml of $15,000 \times g$ supernatant, 9 ml of ice cold 1.15% KCl was added. The samples were then centrifuged at $105,000 \times g$ as previously described. The $105,000 \times g$ supernatant was discarded and 5 ml distilled water was added. The pellet was then resuspended in the water and transferred to a 25 ml stoppered volumetric flask. Distilled water was added to make 25 ml and the flasks were shaken to ensure a uniform protein suspension. Triplicate 1 ml aliquots of the suspension were added to a series of test tubes for protein determination.

The reagents used for this determination were freshly prepared from stock solutions. The copper reagent was prepared by mixing the following stock solutions: 1 volume of 1% copper sulphate, 1 volume 2% sodium potassium tartrate, and 20 volumes of 10% sodium carbonate in 0.5 M NaOH. The dilute Folin-Phenol Reagent,

was prepared by diluting 1 volume of Folin-Phenol Reagent with 10 volumes of distilled water. To each of the test tubes containing 1 ml aliquot of protein, 1 ml of copper reagent was added, the contents were mixed, and allowed to stand for 10 min before 3 ml of dilute Folin-Phenol Reagent was added to each of the test tubes. After thorough mixing, the mixture of protein and reagents was heated in a water bath for 10 min at 50°C. The optical density of each of the samples was then read, using a Unicam spectrophotometer. As a control, aliquots of known concentration of bovine serum albumin (BSA) were used.

A standard curve was prepared, using various known concentrations of Bovine serum albumin, treated similarly to the samples. The optical density versus concentration of serum albumin ($\mu\text{g/ml}$) was plotted and the resulting curve was used to determine the protein concentration of the samples.

G. Determination of Plasma Corticoid Levels

The competitive protein binding technique was used to measure the total plasma corticoids (corticosterone, hydrocortisone). The procedure used was similar to that described by Jeffry and Noujaim (204). The transcortin solution was prepared by diluting 1.25 ml of rat plasma with distilled water to 25 ml. Approximately $1\mu\text{Ci}$ ^3H -cortisol (activity 6 Ci/mM) was added to the solution. It was allowed to equilibrate at room temperature for one hour. Duplicate aliquots of 0.50 ml of the transcortin ^3H -cortisol solution were counted by means of liquid scintillation spectrometry to determine the exact activity of the solution.

The plasma samples obtained from uremic and control rats were assayed for corticosterone as follows: 0.2 ml aliquots of plasma were placed into centrifuge tubes, followed by 0.1 ml water and 1.0 ml 95% ethanol. As a running control for extraction efficiency,

several duplicate samples from above the 0.1 ml water were replaced with 0.1 ml cortisol solution (10 to 20 ng of Cortisol). These samples were shaken for 2 min on the mechanical shaker, after which they were centrifuged for 10 min at 2000 rpm. The supernatant was collected with individual pasteur pipettes. The protein pellet in the test tube was resuspended with a fresh 1 ml aliquot of 95% ethanol and after centrifugation, the two supernatants from each sample were combined. The samples were hydrolyzed and the residue, containing the steroids, was redissolved in 2 ml of distilled water.

Along with each assay run, a fresh series of standards were also prepared. From a stock solution of $10\mu\text{g/ml}$ cortisol, standards were prepared by diluting 0.1, 0.3 and 0.6 ml of the standard stock solution to 100 ml with distilled water to give 10 to 60 ng of cortisol standard working solution. Then, in duplicate, 1 ml of water (0 ng cortisol) and 1 ml aliquots of each of the standard solutions were placed into separate centrifuge tubes. One ml of each of the samples to be assayed was placed into similar centrifuge tubes. To each of the standards and samples, 1 ml of equilibrated protein-isotope solution was added. The tubes were placed into a water bath and incubated for 5 min at 45°C under gentle agitation. The tubes were then cooled in an ice bath for 15 min and 60 mg Fuller's Earth (premeasured) was added to each tube. They were mechanically shaken for 2 min and cooled for a further 10 min in an ice bath. The assay tubes were centrifuged for 10 min at 2000 rpm and the supernatant was collected without stirring the pellet. The supernatant was placed into fresh centrifuge tubes and re-centrifuged to ensure that no Fuller's Earth particles were in suspension. Aliquots of 0.5 ml were transferred to liquid scintillation vials containing 10 ml of Bray's solution (205). The samples were dark adapted and counted in a Picker Liquimat 220, using external standard ratio for quench correction. A series of prepared quench correction standards were also counted and the resulting

data was used to determine the disintegration per minute (dpm) of the samples. From the calculated dpm of each of the cortisol standards and the samples a 1/dpm value was determined. A graph of cortisol (corticosterone) concentrations versus the 1/dpm value was plotted and from this curve, the concentration of the plasma corticoids was determined.

H. Serum Calcium Determination.

Serum calcium was determined by atomic absorption spectrometry (206). A solvent vehicle was prepared by dissolving 5.0 g LaCl_3 , 60 ml n-butanol and 0.2 ml octanol in 0.1 N HCl to give a final volume of 1 litre (206). Calcium standards were prepared from a CaCl_2 stock solution in concentrations of 0, 2, 5, 8, and 10 $\mu\text{g/ml}$ by dilution with the solvent vehicle. Serum samples were prepared for analysis by diluting 0.2 ml of serum to 2.0 ml with LaCl_3 solution. The absorption of each sample was then determined by means of a Beckman 290 Atomic Absorption spectrometer. The concentration of serum calcium was determined by using the standard curve prepared from the calcium standards (Beckman Instruments).

IV. RESULTS

A. Preliminary Assessment of Experimentally Induced Uremia

1. Evaluation of Surgical Techniques Used

a. Renal Occlusion

Contralateral nephrectomy after renal occlusion resulted in an initial rise in the blood urea nitrogen (BUN). The BUN was followed over a period of time during which there was a progressive lowering of the BUN values until they returned to near normal levels (Table I). There was a steady weight gain in all the animals which paralleled those values of the control rats (Table II). However, there was a significant increase in water consumption. Within one week of contralateral nephrectomy, three animals from the 60 minute group and one from the 40 minute group died. Postmortem examination revealed that the remaining kidney, in each case, was pale and contracted, indicating renal shut down.

b. Selective Renal Artery Ligation

These rats demonstrated an initial rise in BUN levels after contralateral nephrectomy (Table I). However, over a period of time, the BUN levels returned to normal. Weight gain (Table II) paralleled that of control animals and again there was an increased consumption of water. Three animals died within one week of contralateral nephrectomy. Again, pale ischemic kidneys were observed in each case.

c. Partial or Sub-total Nephrectomy

After the contralateral nephrectomy was performed, there was a very significant elevation of BUN levels, which remained elevated (Table I). There also was a very significant reduction of weight gain in these animals (Table II). The survival rate in this group was very high. Almost all animals survived longer than one month, although animals which had had more than 70% of their left renal mass removed appeared less likely to survive for longer periods. There was a marked increase in water consumption; in fact, these animals drank twice the quantity consumed by the controls.

TABLE I. THE INFLUENCE OF VARIOUS METHODS OF INDUCTION
OF UREMIA ON THE BUN LEVELS (mg% $\pm$ s.e.)

PROCEDURE	*1st week	2nd week	6th week	10th week	16th week
Occlusion 30 min. (n=8)	43.7 $\pm$ 3.4	31.2 $\pm$ 2.2	29.8 $\pm$ 3.3	28.2 $\pm$ 2.6	--
Occlusion 45 min. (n=7)	41.9 $\pm$ 3.2	34.2 $\pm$ 3.2	24.4 $\pm$ 2.0	30.5 $\pm$ 1.6	--
Occlusion 60 min. (n=5)	34.9 $\pm$ 6.0	36.9 $\pm$ 3.8	34.3 $\pm$ 3.2	35.4 $\pm$ 2.3	--
Partial Ligation (n=5)	36.6 $\pm$ 2.4	39.4 $\pm$ 5.3	41.5 $\pm$ 7.0	41.8 $\pm$ 8.6	--
Polar Ligation (n=7)	61.3 $\pm$ 5.6	62.6 $\pm$ 5.7	54.3 $\pm$ 5.5	51.2 $\pm$ 7.0	--
Gelfoam Method (n=9)	61.8 $\pm$ 2.6	70.7 $\pm$ 3.4	68.9 $\pm$ 5.0	62.0 $\pm$ 5.4	*71.5 $\pm$ 14.7
Control (n=8)	20.1 $\pm$ 1.2	20.5 $\pm$ 1.7	18.8 $\pm$ 0.7	18.8 $\pm$ 1.1	18.0 $\pm$ 1.0

*after contralateral nephrectomy

Normal range is 15 - 25 mg/100 ml

TABLE II. THE INFLUENCE OF VARIOUS METHODS OF INDUCTION
OF UREMIA ON THE BODY WEIGHT OF THE RAT (gramts.e.)

PROCEDURE	*1st week	2nd week	6th week	10th week	16th week
Occlusion 30 min. (n=8)	261± 6.4	289± 9.4	378±17.1	441±26.9	--
Occlusion 45 min. (n=7)	256± 6.5	291± 8.2	387±11.5	436±13.5	--
Occlusion 60 min. (n=5)	254± 4.8	295±11.2	405±24.0	472±43.0	--
Partial Ligation (n=5)	265± 4.5	293± 7.2	--	--	--
Pqlar Ligation (n=7)	249±12.7	275±10.4	336±10.6	371±21.4	--
Gelfoam Method (n=9)	239± 9.1	263± 9.5	323± 9.8	347±17.4	394±20.3
Controls (n=8)	268±11.1	297±13.9	385±16.0	432±18.7	650±2.5

*after contralateral nephrectomy

For some acute uremic experiments, subtotal and contralateral nephrectomy was performed simultaneously, using identical techniques to the two stage operation; the 6 day survival rate was 25% to 30%. The surviving animals would lose at least 20% to 25% of their body weight, as well as show little interest in food for several days (2-3) post-operatively. All rats that died after undergoing this surgery were anuric, indicating shut down of the remaining renal mass.

2. Occurrence of Pathophysiological Changes in Sub-totally Nephrectomized, Chronically Uremic Rats

Sixteen weeks following contralateral surgery, the uremic animals exhibited a very significant reduction in body weight (Table II), to the point where their weight was approximately 60% of normal same age controls. Water consumption was at least double that of the controls. The uremics seemed more sedated and less active than control rats, perhaps exhibiting some of the neurological manifestations one finds in human uremic individuals. It was also observed that uremic rats had severe blood clotting problems, commencing shortly after contralateral nephrectomy. One final observation that was made on a number of chronic uremic rats was that after 8-12 weeks, swelling of the radio-carpal joint was often observed, signifying the existence of rickets in the animals, with perhaps reduced mineralization of bone.

The bones, specifically the femurs, of uremic and controls were examined by x-ray radiography. By inspection of the photoradiographs (Fig. 3) it can be observed that the uremic femurs resemble the younger same weight controls more closely than the same age controls. Not only were the uremic bones smaller but they also exhibited decreased radio-density as compared to the same age controls. Maturation of the epiphysis was not evident in the uremic rat femur. A subjective observation made while removing the femurs was that in the uremic animal, the bone was softer than the same age control. The uremic bone could be cut with a scalpel fairly easily, while the control animal femurs were very hard. Reduced calcium

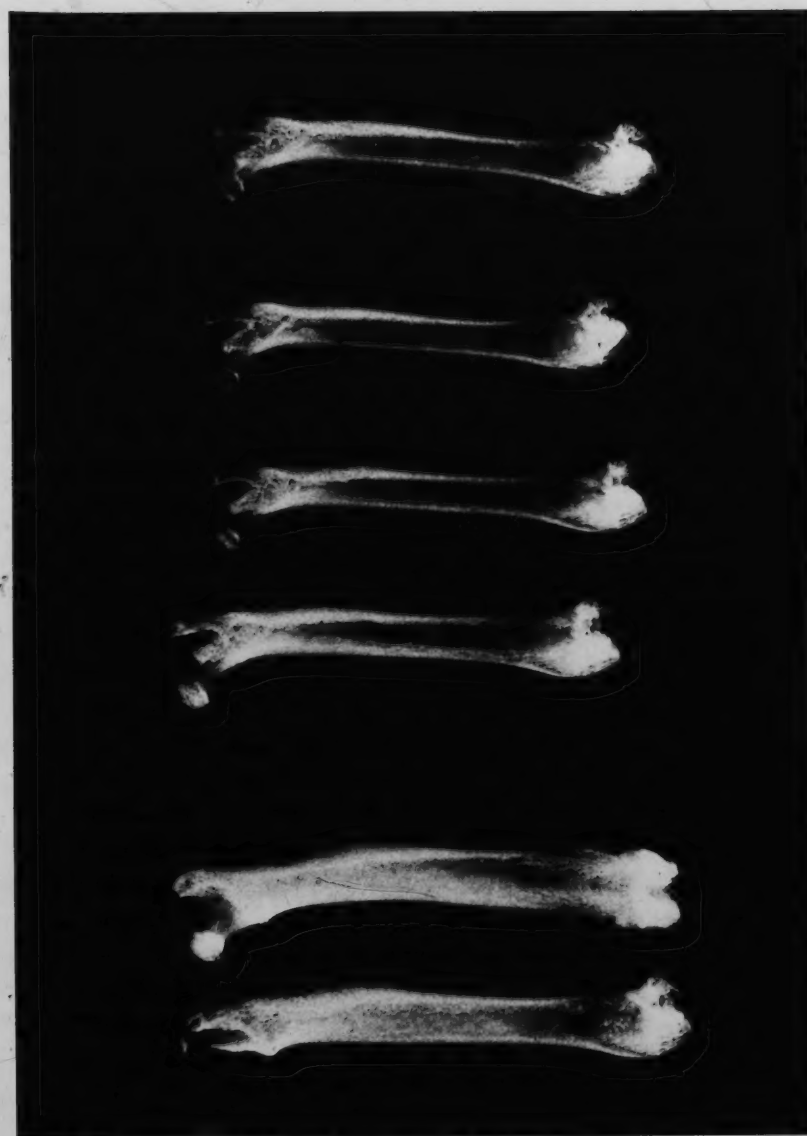


Figure 3. Photoradiographs of the X-ray Radiogram of Femurs Taken from the same age Control (A), Uremic (B) and same weight Control (C) Rats, sixteen weeks after subtotal and contralateral nephrectomy.

content was detected in the ash of uremic femurs which had been ashed in a muffle furnace at 800°. The uremic bones had a significantly lower content of ^{45}Ca present than controls after an oral dose of calcium-45. The uremic bone contained 145 ± 21 cpm per gram wet bone ($n=5$) whereas the control femurs ($n=5$) contained 219 ± 29 cpm per gram wet weight of bone (207).

Further histopathological investigation of calcified and decalcified bone tissue (by Dr. T. Smitka of the University of Alberta Hospital), such as ribs and spine, indicated that the bone development was dormant (208).

When the renal tissue was examined, it was found that the residual portion of the kidney had hypertrophied to a size where its weight was similar to the two healthy kidneys in control animals. Light microscopic examination of histological sections of the residual renal mass indicated that the observed hypertrophy of the kidney may, at least partially, have been due to the hypertrophy of the entire nephron unit. The Bowman capsule was greatly enlarged in the uremic rat kidney, compared to that in normal renal tissue (Fig. 4, 5). Also, the lumen of the convoluted tubules was noticeably increased in diameter. The cells of the convoluted tubules were low and flattened, instead of cuboidal, as they are in the normal state. This may have been due to the compensatory diuresis which occurs in uremia.

Examination of hepatic tissue also demonstrated a number of significant changes (Fig. 6, 7). Light microscopic examination indicated the presence of vacuoles in uremic hepatocytes. Electron microscopic examination elucidated some significant changes in the ultra-structure of the uremic hepatocyte (Fig. 8, 9). There appeared to be an abundance of rough endoplasmic reticulum and increased glycogen content. Low power observation of tissue sections from experimental animals showed an increase in the occurrence of rough

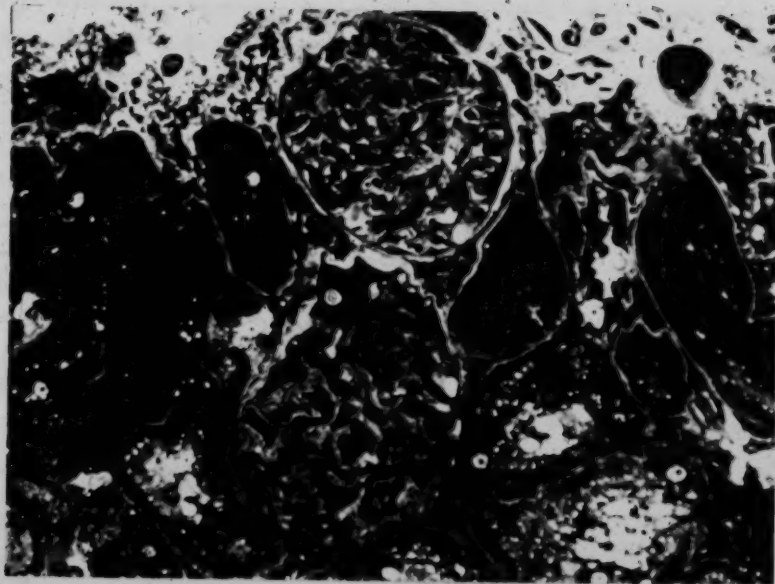


Figure 4. Photomicrograph of Normal Kidney Tissue X 750.

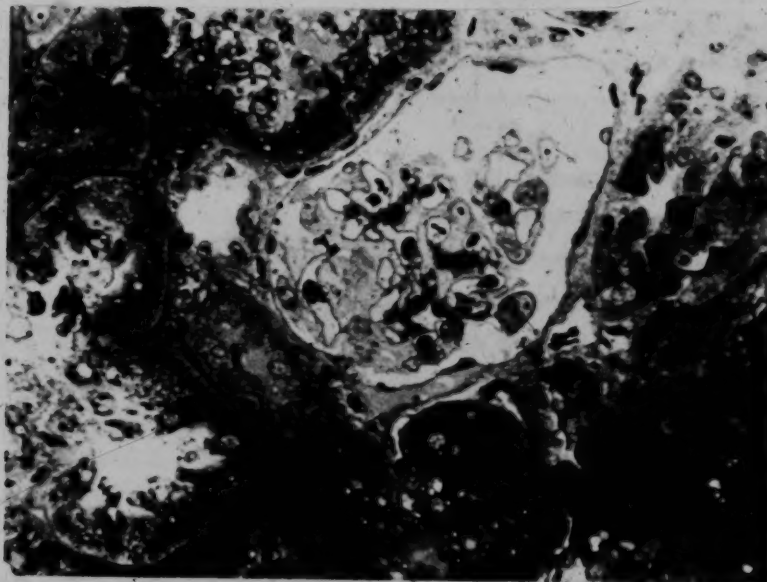


Figure 5. Photomicrograph of Uremic Kidney X 750

Note Hypertrophied Bowman's Capsule and Dilated Tubules
in Uremic Tissue.



Figure 6. Light Photomicrograph of Normal Rat Liver X 1250

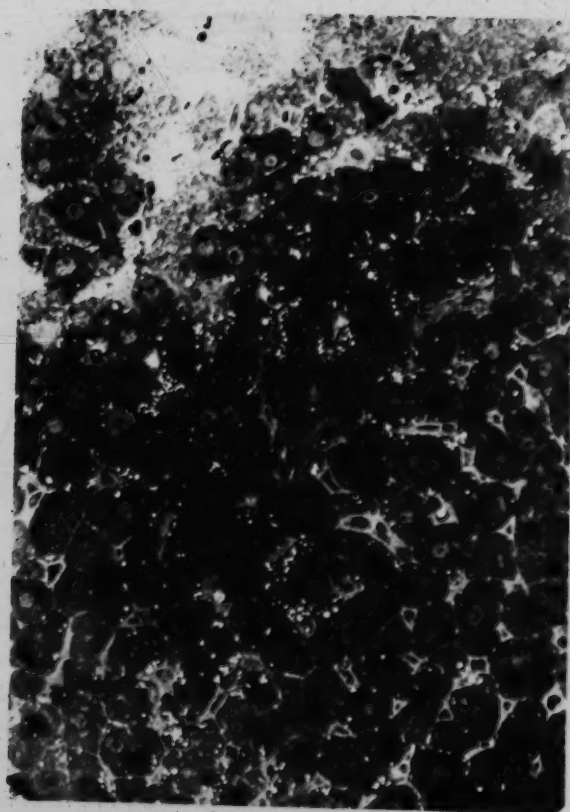


Figure 7. Light photomicrograph of uremic rat liver

Note the presence of vacuoles in uremic hepatocytes X 1250.

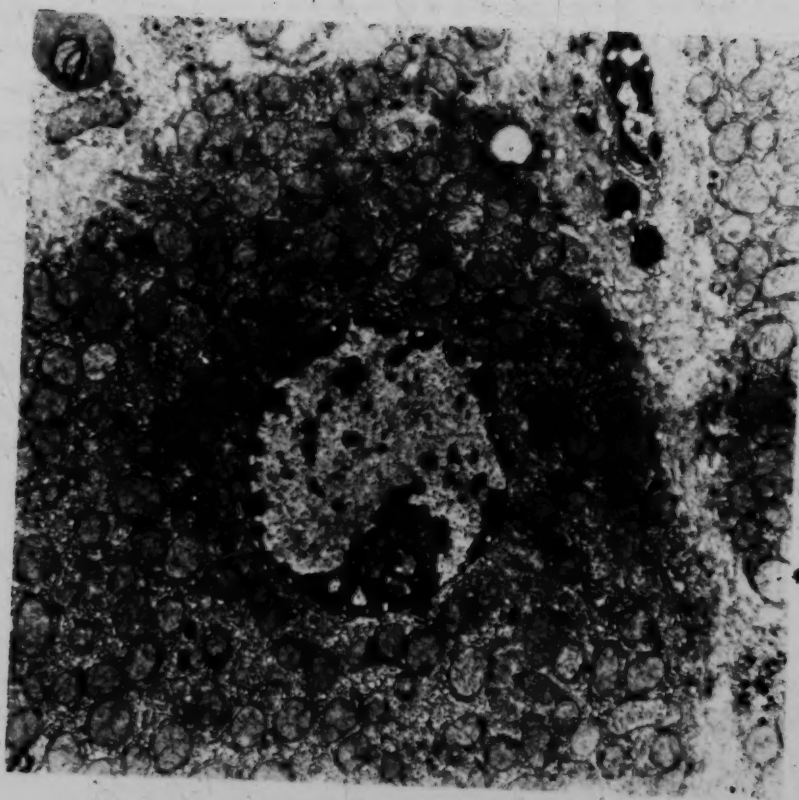


Figure 8. Electron photomicrograph of normal liver X 8,463.

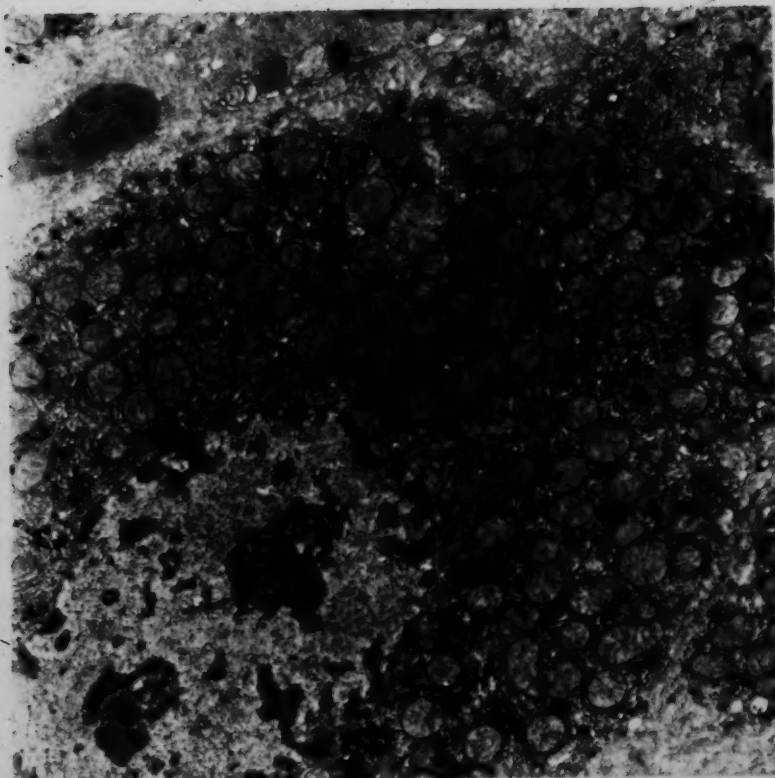


Figure 9. Electron photomicrograph of uremic liver.

Note the abundance of darkly stained glycogen granules in the uremic hepatocytes, as well as increased rough endoplasmic reticulum X 8,463.

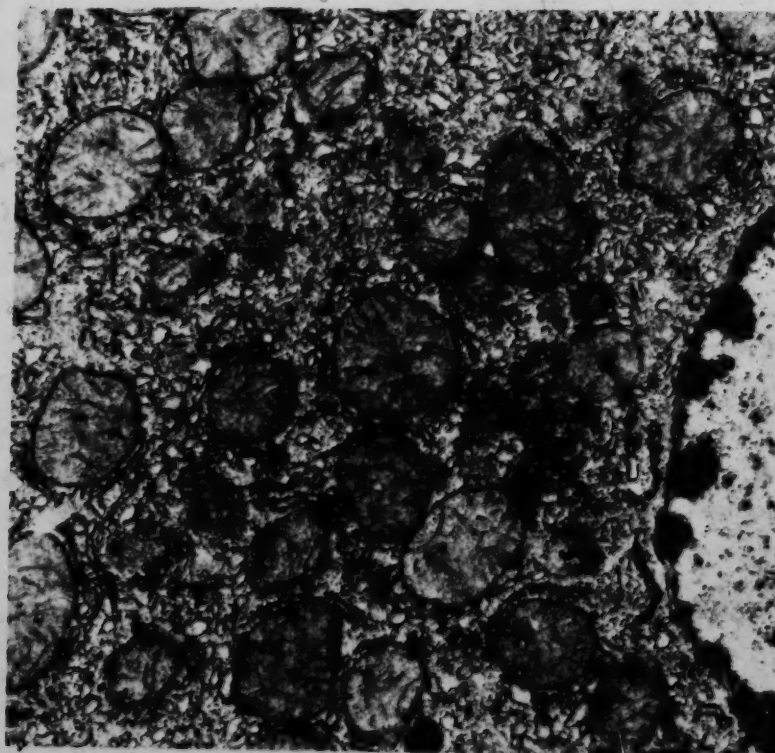


Figure 10. Electron photomicrograph of normal liver X 23,650.

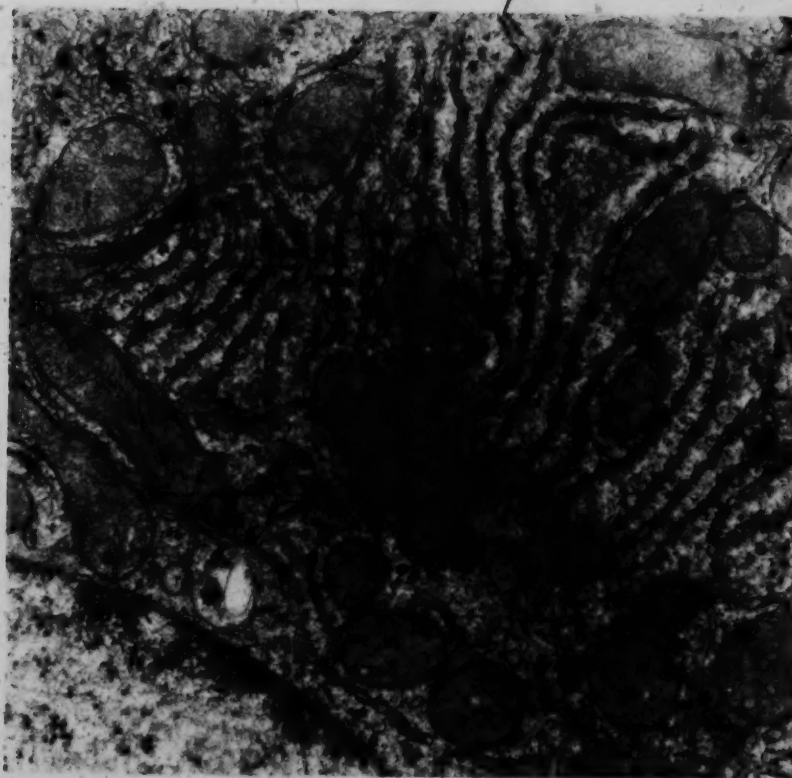


Figure 11. Electron photomicrograph of uremic rat liver.

Note the increased amount of rough endoplasmic reticulum /
X 23,650.

endoplasmic reticulum (RER) in the perinuclear region in particular, while the glycogen was largely confined to the cell periphery. The morphology of the RER in uremic tissue appeared entirely normal and its increase in quantity would indicate enhanced synthesis of protein. No significant differences could be seen in the density of the ribosomes attached to the RER, or occurring free in the cytoplasm (Fig. 10, 11).

B. Effect of Uremia on In Vivo Metabolism of ^{14}C -Vitamin D_3

1. Distribution of Radioactivity in Uremic and Control Rats

After the intraperitoneal dose of $0.5 \mu\text{Ci}$ ethanolic ^{14}C -Vitamin D_3 , the $100 \mu\text{l}$ blood aliquots were collected and scintillation counted for activity. The graphic representation of blood activity in uremic and control animals is shown in Fig. 12. It can be seen that that radioactivity representing Vitamin D_3 and its metabolites in normal rats reached its maximum value in three hours and then stayed fairly constant at that level. In contrast, the activity in the uremic animals' blood samples rose rapidly over a six hour period, finally reaching a peak at 12 hours. Furthermore, it should be noted, that after 6 hours the blood radioactivity in the uremic animals was at least three times the value of that found in controls.

Examination of other tissues revealed that there was a significant increase in radioactivity, representing both the labeled Vitamin D_3 and its metabolites, in the uremic tissues as compared to those levels in tissues of same weight control animals. The uremic liver radioactivity was 6830dpm/g versus 2720dpm/g in the liver of controls. In renal tissue a smaller difference was observed, i. e., $14,500\text{dpm/g}$ in uremics versus $11,300\text{dpm/g}$ in controls. There was approximately a two-fold difference in the small intestine, between the uremics and the controls, namely $3,970\text{dpm/g}$ compared to $2,260\text{dpm/g}$. (Table IV).

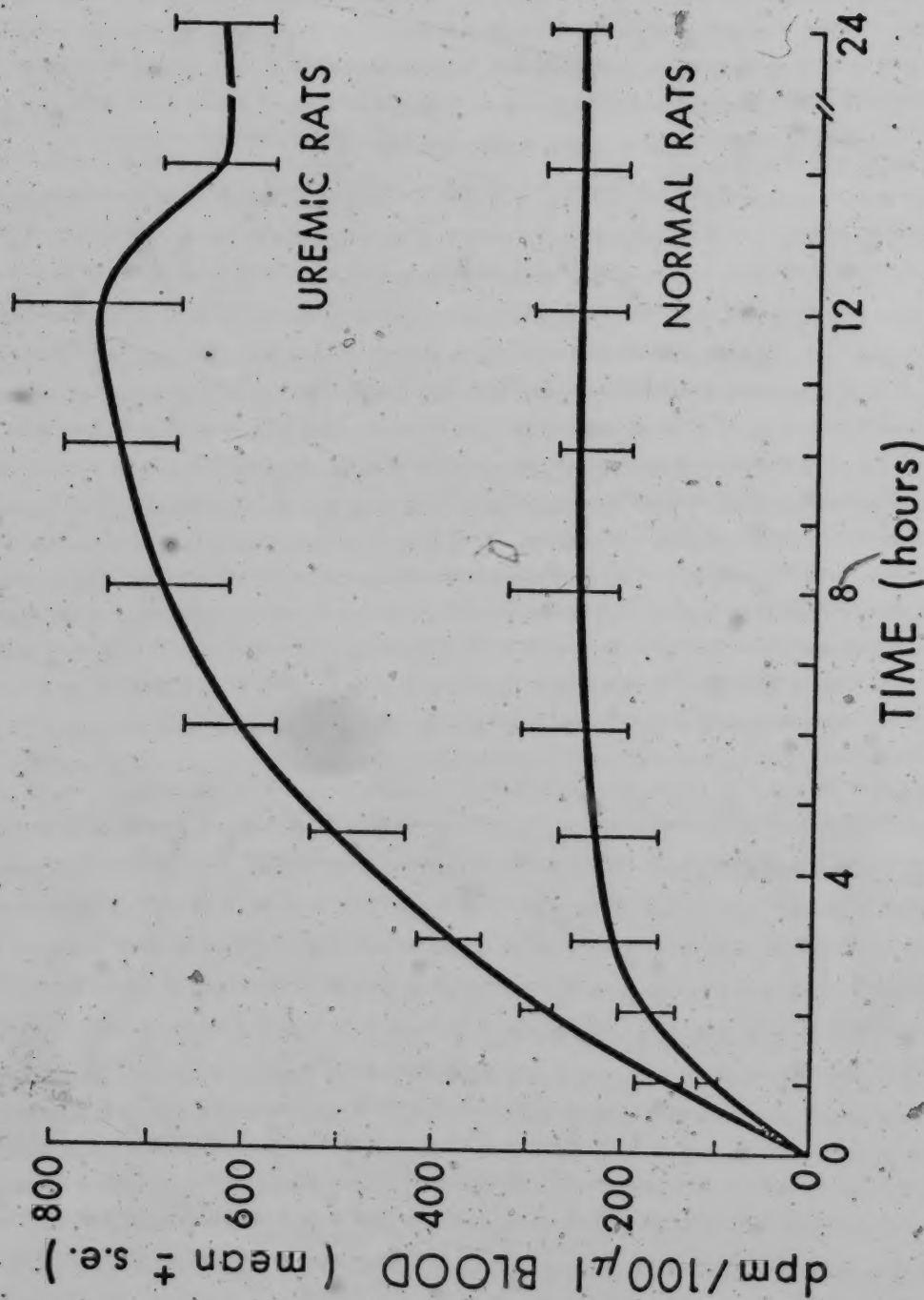


Figure 12:

Determination of blood radioactivity in uremic and control rats after an intraperitoneal injection of ^{14}C -Vitamin D_3 . (0.5 μCi , 240 I. U.) Blood samples (100 μl) were obtained at specific time intervals and radioactivity determined. Each determination is the mean of 6 control and 6 uremic rats.

TABLE III

DISTRIBUTION OF ^{14}C -LABELED VITAMIN D_3 AND METABOLITES IN PLASMA, 24 HOURS
 AFTER INTRAPERITONEAL INJECTION OF $100\mu\text{l}$ ETHANOLIC ^{14}C -VITAMIN D_3 (0.5 μCi)
 - EXPRESSED AS % OF TOTAL RADIOACTIVITY.

TISSUE	RAT	METABOLITE				TOTAL ACTIVITY ^a
		I ^c	III ^c	IV ^c	Va ^c Vb ^c V ^c	
PLASMA	CONTROL ^d	7.0	81.0	10.6	TRACE 2.0	--
	UREMIC ^d	13.0	58.6	14.8	5.3 3.7	4.4
	UNILATERAL ^d NEPHRECTOMY	11.7	56.9	16.7	5.3 2.7	4.1
PLASMA	CONTROL ^e	7.3 $\pm$ 1.2	86.9 $\pm$ 3.8	10.0 $\pm$ 1.3	TRACE	TRACE --
	UREMIC ^e	7.6 $\pm$ 1.9	81.8 $\pm$ 4.1	10.7 $\pm$ 1.0	TRACE	TRACE TRACE

a - Disintegrations per minute

b - Volume of plasma

c - I = esterified Vitamin D

III = Vitamin D

IV = 25(OH) D_3

Va = 24, 25(OH) D_2D_3

Vb = not identified

V = 1, 25(OH) D_2D_3

d - pooled plasma from three animals

e - mean values from three separate determinations
 $\pm$ S. D.

2. Chromatographic Analysis of Plasma and Other Tissues of Uremic, Unilateral Nephrectomized, and Control Animals

After the chromatographic analysis of the various tissues was completed, it became evident that there was a qualitative, as well as quantitative, difference in the appearance of the various metabolites of Vitamin D_3 (Fig. 13) in these three types of animals. The uremic plasma contained significant metabolic peaks, which represented metabolites more polar than both Vitamin D_3 or $25(OH)D_3$. Using the comparative method of Holick and DeLuca (112), these were tentatively identified as $24,25(OH)_2D_3$, peak Vb and $25,26$ - and/or $1,25-(OH)_2D_3$ (see Table VII). There was little evidence of their presence in the control plasma. However, it was interesting to note that these polar metabolites were also observed in the plasma of the unilaterally nephrectomized animals. However, in the plasma of these rats, while the magnitude of the peaks more polar than $25(OH)D_3$ was less than that observed in the uremic plasma (Fig. 13), the relative abundance of metabolites found in unilateral nephrectomized rat plasma was similar to that of uremic plasma (Table III).

Chromatographic examination of the livers, kidneys and small intestines also revealed quantitative changes in the three groups of animals (Table IV). In the uremic tissues, there were significantly larger quantities of Peak I, III and IV, representing esterified calciferol and its metabolites, Vitamin D_3 and $25(OH)D_3$, respectively. It was also noteworthy that control livers had an esterified peak of Vitamin D representing a relatively larger quantity of the total radioactivity while the uremic and unilateral control livers had a proportionately larger quantity of ^{14}C -calciferol present.

In the kidney and small intestine, the distribution of the three metabolites was similar. However, the uremic tissues again contained significantly larger absolute quantities of each of the metabolites (Table IV).

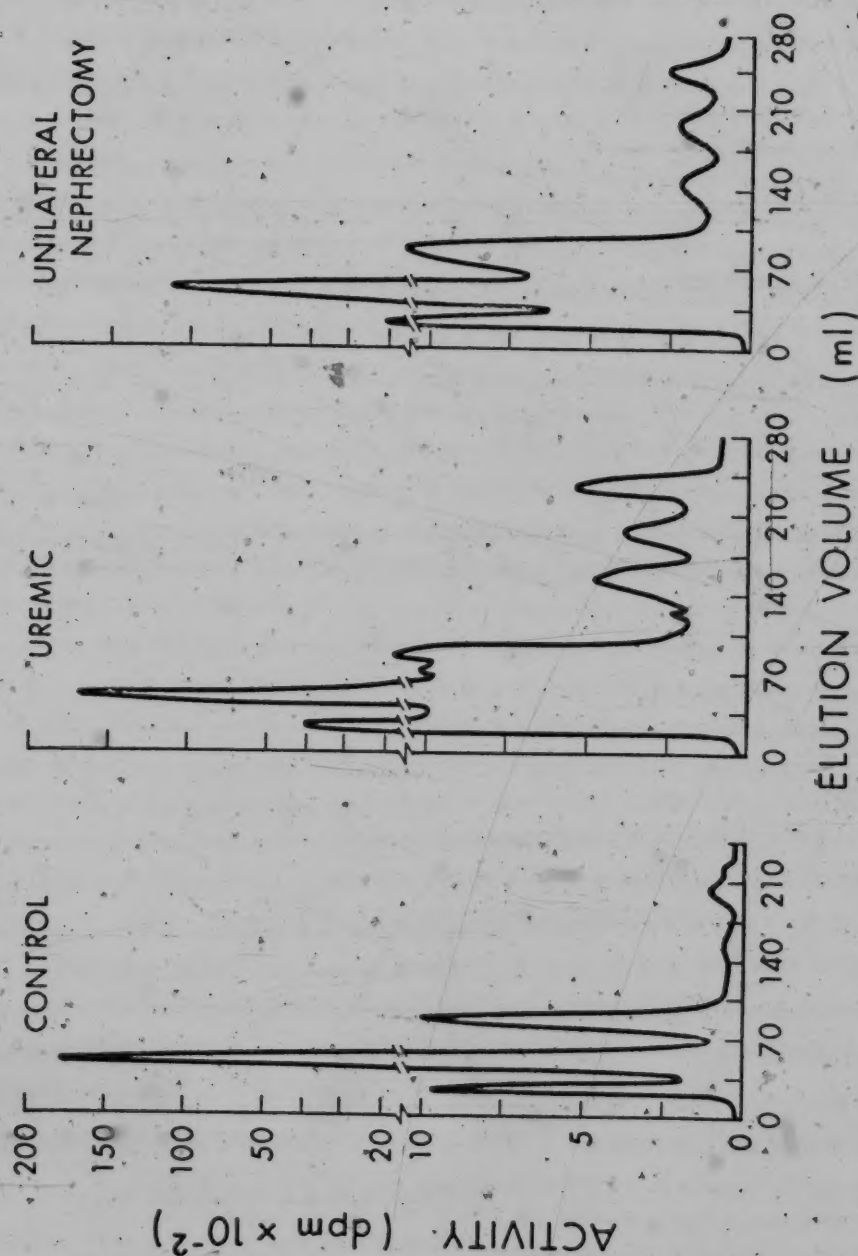


Figure 13: Sephadex LH-20 column (1.3 x 50 cm packed in 65:35 v:v CHCl₃-n-hexane) profile of serum lipid extracts obtained from a. control, b. uremic, and c. unilateral nephrectomized controls given intraperitoneally 0.5 μ Ci (240 I.U.) ¹⁴C-Vitamin D₃, 24 hours previously. Each profile represents the extract of the combined plasma of three rats.

TABLE IV. DISTRIBUTION OF ^{14}C -VITAMIN D_3 AND METABOLITES,
24 HOURS AFTER AN INTRAPERITONEAL INJECTION OF
100 μl ETHANOLIC ^{14}C -VITAMIN D_3 (0.5 μCi)
- EXPRESSED AS PERCENT OF TOTAL RADIOACTIVITY

TISSUE	RAT	METABOLITES			ACTIVITY ^a
		I	III	IV	
LIVER	CONTROL	15.3 $\pm$ 3.9	81.0 $\pm$ 3.8	3.7 $\pm$ 0.6	2,720 $\pm$ 270
	UREMIC	9.5 $\pm$ 0.5	87.3 $\pm$ 1.1	3.2 $\pm$ 0.6	6,830 $\pm$ 730
	UNILAT. NEPHR.	7.4 $\pm$ 0.9	89.7 $\pm$ 1.0	2.8 $\pm$ 0.3	2,826 $\pm$ 240
KIDNEY	CONTROL	10.7 $\pm$ 1.0	87.6 $\pm$ 2.7	1.7 $\pm$ 0.6	11,300 $\pm$ 340
	UREMIC	11.8 $\pm$ 2.7	85.5 $\pm$ 2.4	2.7 $\pm$ 0.7	14,500 $\pm$ 750
	UNILAT. NEPHR.	11.8 $\pm$ 2.0	86.6 $\pm$ 1.8	1.6 $\pm$ 0.2	6,740 $\pm$ 425
SMALL INTESTINE	CONTROL	11.7 $\pm$ 3.4	82.6 $\pm$ 2.3	6.0 $\pm$ 2.1	2,260 $\pm$ 570
	UREMIC	10.5 $\pm$ 0.5	83.4 $\pm$ 1.2	6.1 $\pm$ 1.3	3,970 $\pm$ 142
	UNILAT. NEPHR.	--	--	--	--

^aDPM per gram of tissue

Each value represents the mean and standard error of mean of 4 determinations

C. Effect of Uremia and Vitamin D Deficiency on the Metabolism of an Intravenous Pulse Dose of ^{14}C -Vitamin D_3 and ^3H -25-Hydroxy Vitamin D_3 .

After the administration of an ethanolic (50 μl) mixture of ^{14}C -Vitamin D_3 and ^3H -25(OH) D_3 (0.25 μCi of each) intravenously, to rachitic, uremic and control rats, 100 μl aliquots of blood were examined for radioactivity. The presence of tritium, representing ^3H -25(OH) D_3 and its metabolites, revealed that the time and course was similar in all groups, with the exception that the uremic blood levels were somewhat lower than those of rachitic and control rats (Fig. 14).

The basic time course pattern of carbon 14, representing circulating ^{14}C -Vitamin D and its metabolites, was also similar in rachitic and control rat blood (Fig. 14). In contrast, the uremic group, after displaying an initial drop in blood radioactivity, rose sharply and peaked at about 80 minutes after the intravenous administration of the labeled material. This was then followed by a decline, subsequent to which there was a steady increase in the blood level of the uremic animals, so that after 8 hours, the blood radioactivity was about three fold that of the rachitic or control levels (Fig. 14).

Chromatographic analysis of the plasma from rachitic, uremic and control rats by means of Sephadex[®] LH-20 column chromatography, also demonstrated some significant changes in the metabolic pattern (Table V). In control plasma, 11.9% of the total ^{14}C -radioactivity was represented by ^{14}C -25(OH) D_3 . A significant quantity of ^{14}C -24,25(OH) $_2\text{D}_3$ and some ^{14}C -1,25(OH) $_2\text{D}_3$ (1.5%) was also evident. The ^3H -25(OH) D_3 was further metabolized to ^3H -24,25(OH) $_2\text{D}_3$ (5.4%) and a minute quantity of ^3H -1,25(OH) $_2\text{D}_3$ (0.3%) (Table V). In rachitic animals, ^{14}C -25(OH) D_3 represented 21.2% of the total activity, while smaller quantities of 24,25(OH) $_2\text{D}_3$ (1.7%) and 1,25(OH) $_2\text{D}_3$ (1.4%) were evident. The main metabolite of ^3H -25(OH) D_3 was ^3H -1,25(OH) $_2\text{D}_3$ (2.2%), as one would expect, while a smaller quantity (0.8%) of ^3H -24,25(OH) $_2\text{D}_3$.

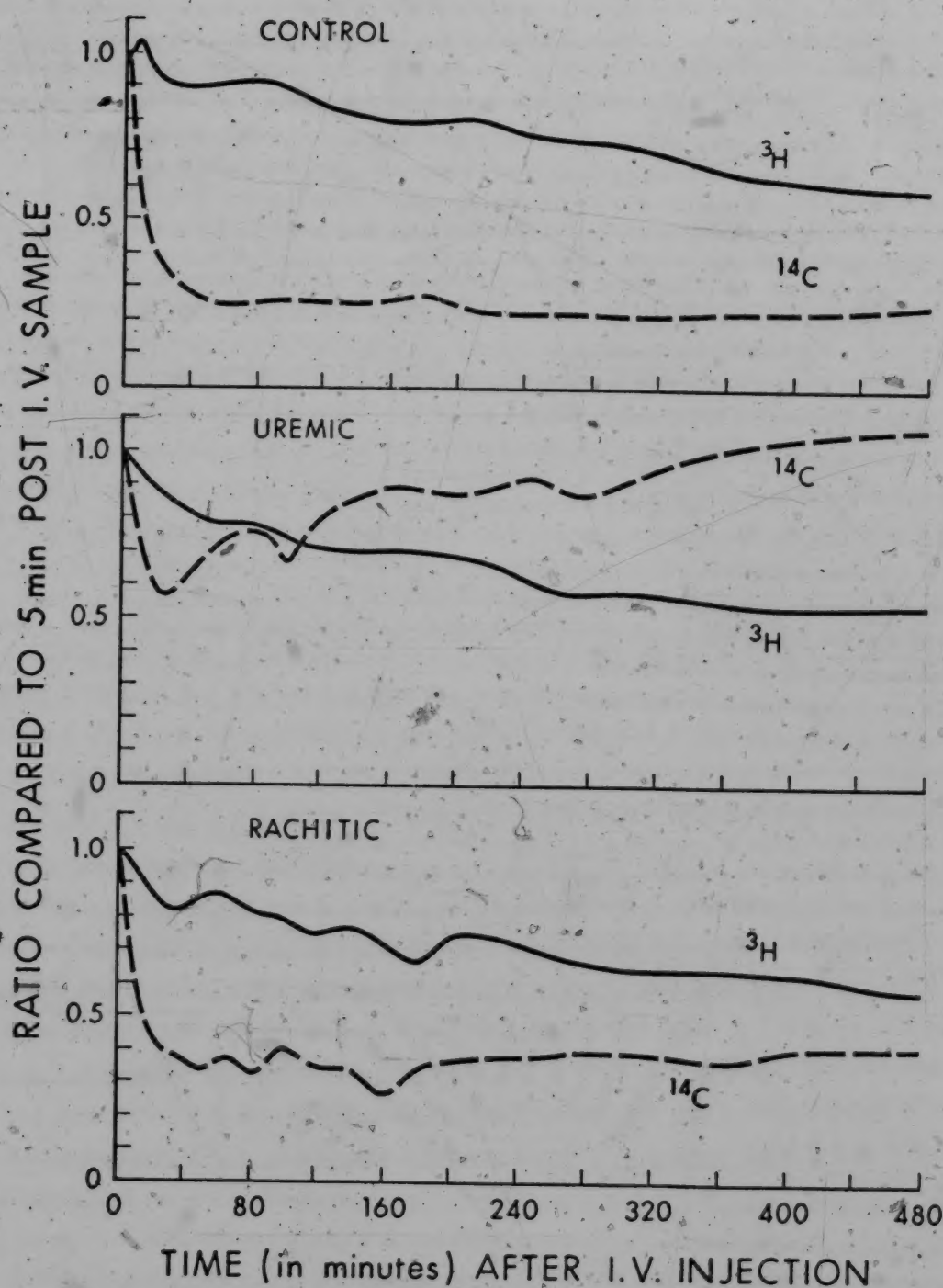


Figure 14: Determination of the radioactivity in 100 μl blood samples in control, rachitic and uremic rats after an intravenous injection of 50 μl ethanol, containing 0.25 μCi each of ^3H -25OHD₃ and ^{14}C -Vitamin D₃. Each value represents the mean of six animals. Note: for values of specific points on curves see page 140.

TABLE V. DISTRIBUTION OF ^{14}C -VITAMIN D_3 AND METABOLITES AS WELL AS ^3H -25-HYDROXY-VITAMIN D_3 AND METABOLITES IN PLASMA OF CONTROL, RACHITIC AND UREMIC RATS AFTER AN INTRAVENOUS INJECTION OF A COMBINED DOSE OF 0.25 μCi EACH OF ^{14}C -VITAMIN D_3 AND ^3H -25-HYDROXY-VITAMIN D_3 IN 50 μl ETHANOL. - EXPRESSED AS PERCENT OF TOTAL PLASMA RADIOACTIVITY.

ANIMAL	ESTER- D_3^a	METABOLITES			TOTAL ACTIVITY	
		VIT- D_3	$25(\text{OH})\text{D}_3$	$24,25(\text{OH})\text{D}_3$	$1,25(\text{OH})_2\text{D}_3$	(DPM)
CONTROL ^{14}C	14.3	69.3	11.9	3.0	0.5	54,000
^3H	0.3		94.0	5.4	0.3	309,200
RACHITIC ^{14}C	27.3	48.4	21.2	1.7	1.4	11,600
^3H	0.3		96.7	0.8	2.2	61,300
UREMIC ^{14}C	12.9	77.4	8.5	1.2	TRACE	11,500
^3H	0.3		98.4	1.2	TRACE	29,400

^aEsterified Vitamin D_3 and Metabolites

Note: Values presented were derived from pooled plasma sample of 6 controls, 4 rachitic and 3 uremic animals.

TABLE VI. TISSUE DISTRIBUTION OF ^{14}C AND ^3H , REPRESENTING ^{14}C -VITAMIN D_3 AND METABOLITES, AS WELL AS ^3H -25-HYDROXYVITAMIN D_3 AND METABOLITES 8 HOURS AFTER INTRAVENOUS INJECTION OF ETHANOL ^3H -25(OH) D_3 AND ^{14}C -VITAMIN D_3 (0.25 μCi OF EACH IN A SINGLE DOSE).

TISSUE	RAT	CARBON-14 ^a	TRITIUM ^a
LIVER	CONTROL	12,133 $\pm$ 1066	840 $\pm$ 104
	UREMIC	12,867 $\pm$ 2756	1,072 $\pm$ 100
	RACHITIC	20,564 $\pm$ 3646	768 $\pm$ 83
KIDNEY	CONTROL	1,993 $\pm$ 258	1,134 $\pm$ 136
	UREMIC	2,343 $\pm$ 166	1,437 $\pm$ 150
	RACHITIC	2,238 $\pm$ 552	1,204 $\pm$ 168

^aActivity Expressed as DPM per gram tissue

^bValues $\pm$ standard error of mean of 6 control, 6 uremic, and 4 rachitic animals.

was also present (Table V). In the uremic plasma, the ^{14}C -25(OH) D_3 was present in a much smaller quantity (8.5%) than in either control (11.9%) or rachitic (21.2%) plasma. The only dihydroxy-metabolite of either ^{14}C or ^3H labeled parent compound was a relatively small amount of 24,25(OH) $_2\text{D}_3$. There was little evidence of 1,25(OH) $_2\text{D}_3$ in the uremic plasma.

Liver and kidney distribution of radioactivity representing metabolites as well as the parent compounds of both ^3H -25(OH) D_3 and ^{14}C -Vitamin D_3 was also investigated. There appeared to be little significant difference in the quantity of radioactivity (either tritium or carbon-14) in the renal tissue on a weight basis (Table VI), among all three groups of animals. However, there were significant differences in the radioactivity in liver tissues. The levels of radioactivity in control and uremic livers were similar, while the carbon-14 activity in the rachitic liver was greater than 1.5 times that of control or uremic liver tissue (Table VI).

D. In Vitro Metabolism of ^{14}C -Vitamin D_3 and ^3H -25-Hydroxy-Vitamin D_3

1. Metabolism of ^{14}C -Vitamin D_3 In Vitro

The in vitro study of ^{14}C -Vitamin D_3 metabolism by liver homogenate preparations proved to be somewhat disappointing. In the initial in vitro studies of ^{14}C -Vitamin D_3 metabolism by both liver homogenates and 9000x g supernatant a shoulder or small peak appeared on the Sephadex LH-20 chromatogram (Fig. 15). However it was difficult to observe any significant differences in the rate of hydroxylation of ^{14}C -labeled calciferol in uremic or rachitic liver preparations, when compared to control incubates. The addition of malate to the incubates did not appear to enhance the production of hydroxylated Vitamin D , as the chromatograms of control, uremic, and rachitic rat liver incubate extracts (Fig. 15). Increasing the liver homogenate or 9000 x g

TABLE VII. DETERMINATION OF ELUTION VOLUMES FOR VITAMIN D₃
AND ITS METABOLITES USING BOTH LABELED AND UNLABELED
REFERENCE COMPOUNDS USING A 1.3 x 50 cm
SEPHADEX LH-20 COLUMN AND CHLOROFORM:HEXANE
(65:35, v:v)

COMPOUND	ELUTION VOLUME (ml)	
VITAMIN D ₃	44 ± 3.9 ^c	(44) ^b
25(OH)D ₃	67.3 ± 4.8	(65)
24,25(OH) ₂ D ₃ ^a	154.3 ± 8.2	(152)
1,25(OH) ₂ D ₃	262.0 ± 16.1	(254)

^a Determined from normal chick renal incubate using ³H-25(OH)D₃ as substrate

^b Published values. Ref. 112

^c Standard deviation of mean - 6 determinations for each value

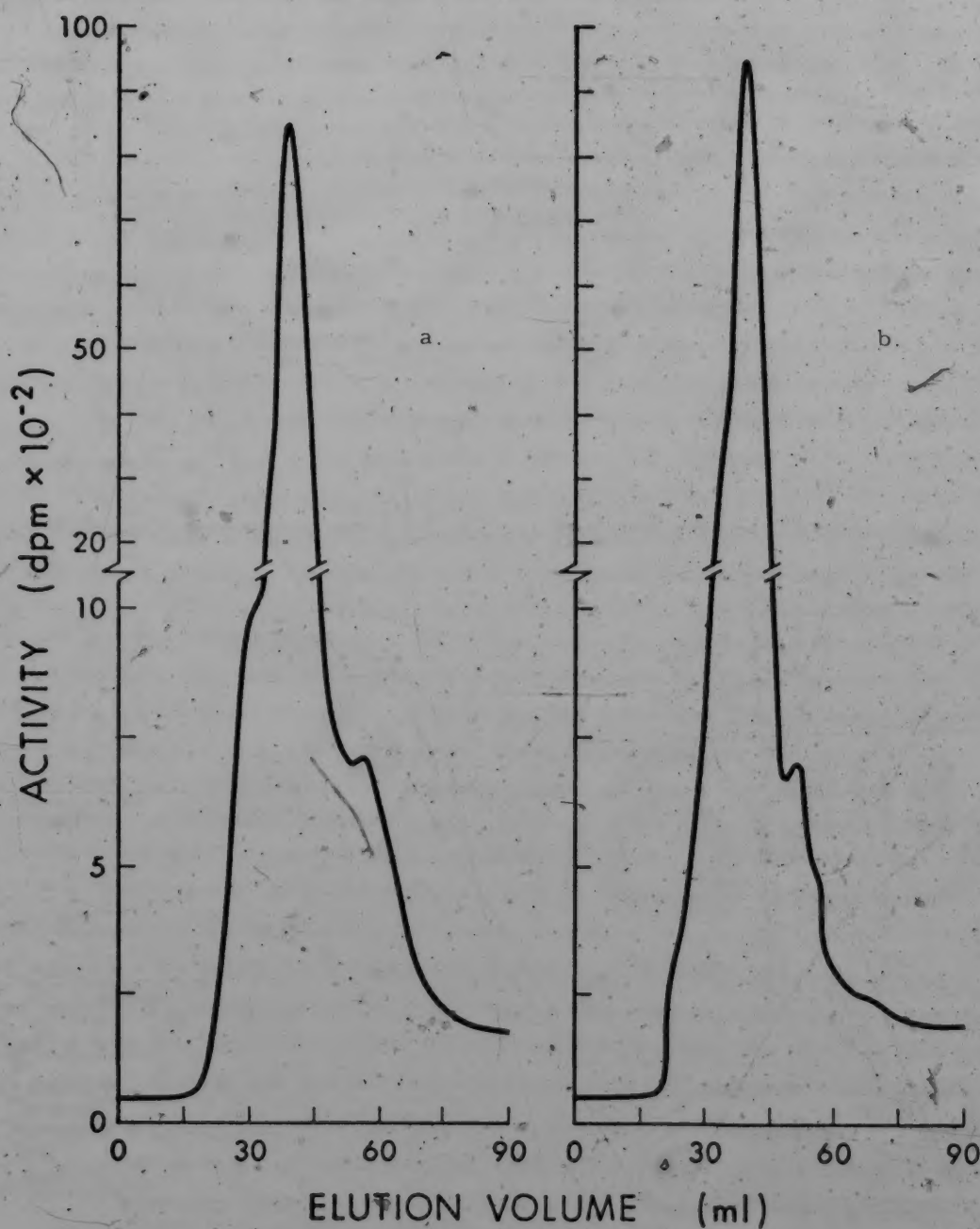


Figure 15: Representative Sephadex LH-20 (1.3 x 50 cm column using CHCl_3 -n-hexane 65:35) chromatographic profile of extracts of a. 20% rat liver homogenate (2 ml) and b. 9000 x g supernatant 2 ml, incubated with $0.2 \mu\text{Ci } ^{14}\text{C-4-Vitamin D}_3$ for 2 hours. Representative of 6 control, 6 rachitic, and 6 uremic animals.

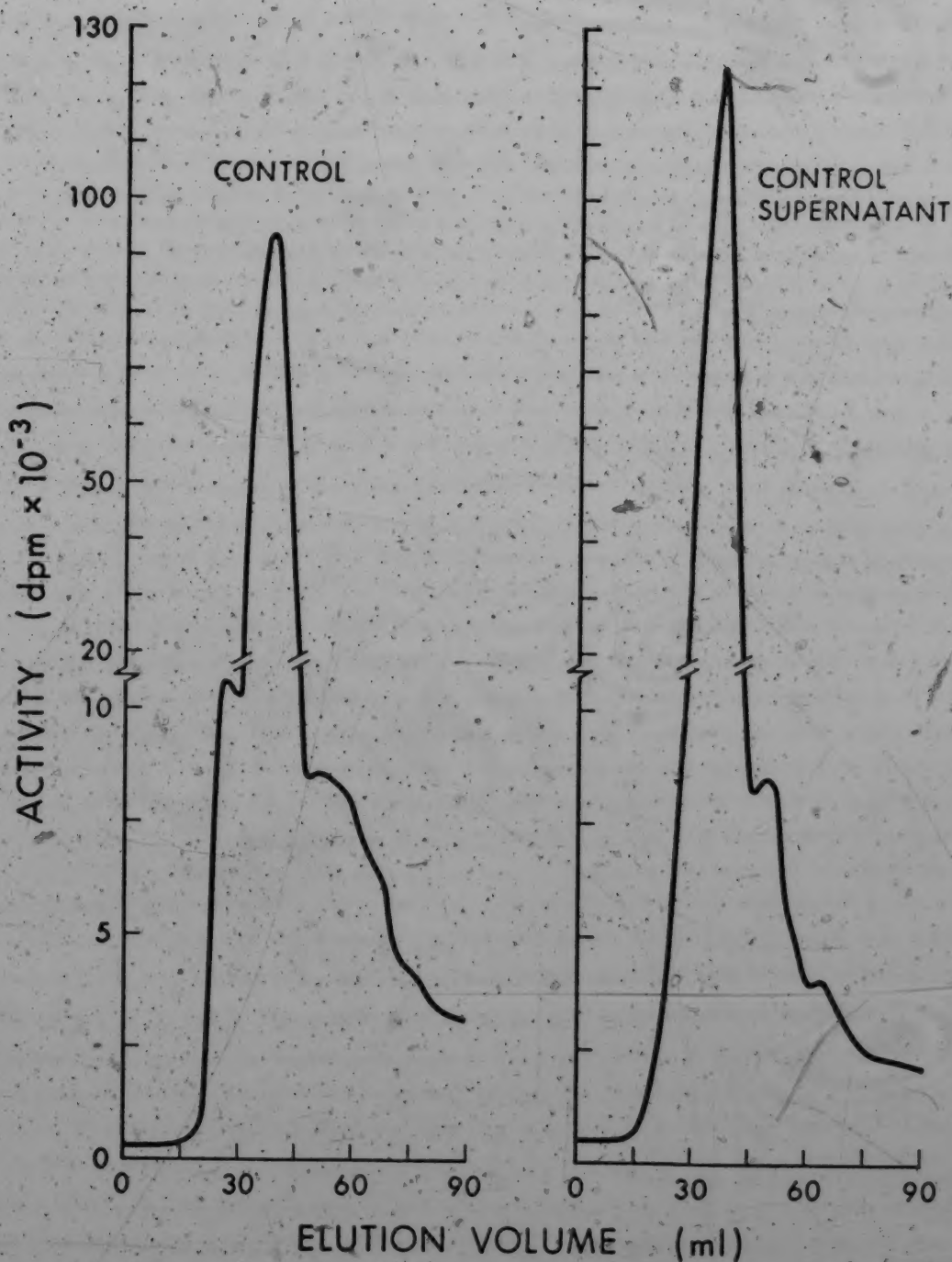


Figure 16: Representative Sephadex LH-20 (1.3 x 50 cm column using CHCl_3 -n-hexane 65:35, v:v) chromatographic profile of extracts of a. 20% rat liver homogenate (6 ml) b. 9000 x g supernatant (6 ml), incubated with $0.2 \mu\text{Ci } ^{14}\text{C-4-Vitamin D}_3$ for 2 hours. Representative of 6 control and 6 uremic animals.

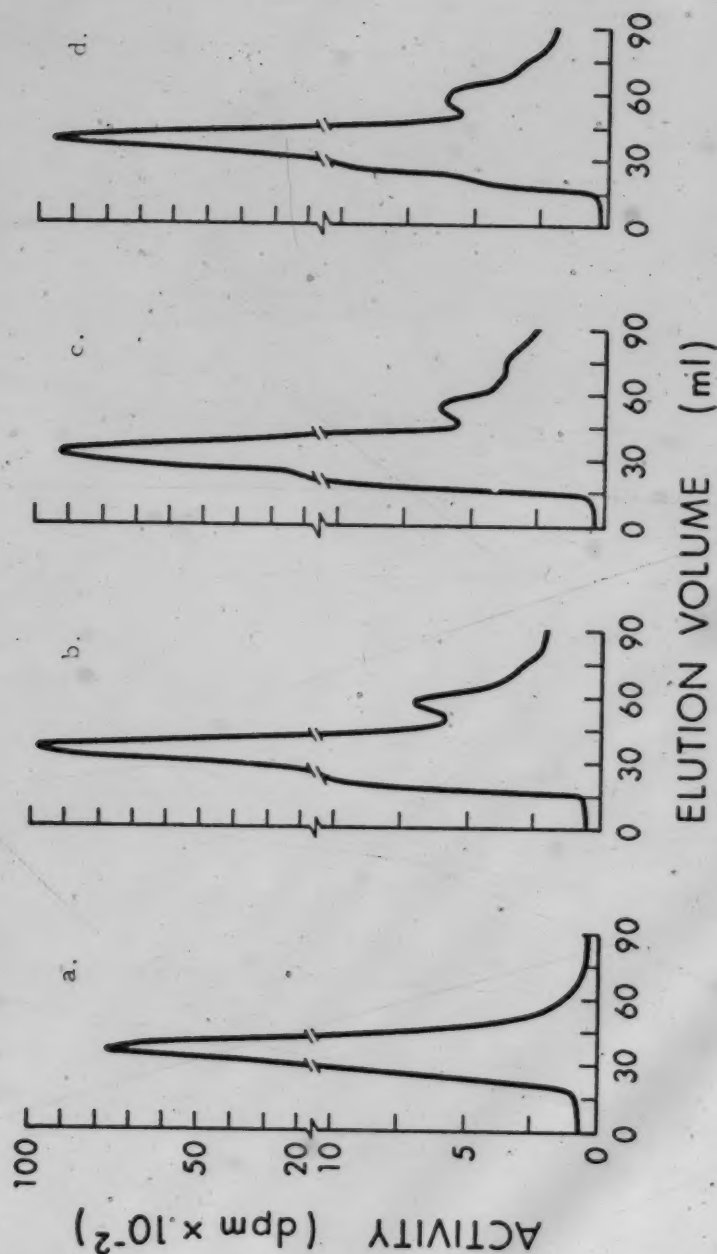


Figure 17: Representative Sephadex LH-20 (column 1.3 x 50 cm CHCl_3 -n-hexane 1:1) chromatographic profiles of a. substrate only, b. controls, c. rachitic and d. uremic rat liver incubates using 25% liver homogenates prepared in 0.25 M sucrose. 5 ml homogenate was used. Representative of 6 animals in each group.

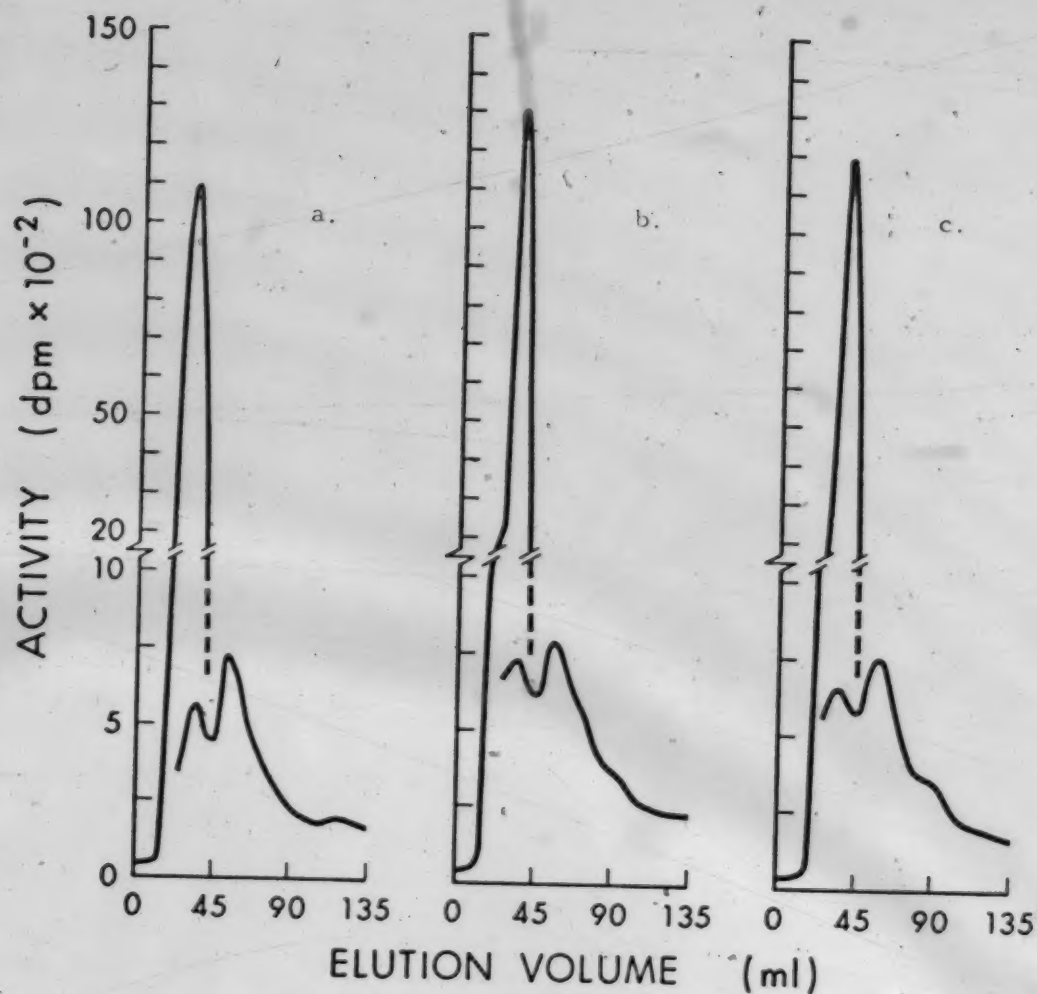


Figure 18: Sephadex LH-20 chromatographic profile (1.3 x 50 cm column, CHCl₃-n-hexane 1:1, v:v) of a. control, b. rachitic and c. uremic rat liver homogenate incubated with ¹⁴C-Vitamin D₃. The region of 25(OH)D₃ was collected, concentrated and rechromatographed.

TABLE VIII DETERMINATION OF R_f VALUES OF BOTH LABELED AND UNLABELED VITAMIN D_3 AND 25-HYDROXY-VITAMIN D_3 USING CHROM-AR-1000 AND VARIOUS SOLVENT SYSTEMS.

SOLVENT SYSTEM	COMPOUND		
	VIT. D_3	25(OH) D_3	1,25(OH) $_2D_3$
1. ETHYL ACETATE 100%		0.79 ± 0.02^c	$(0.4)^a$
2. ETHYL ACETATE- HEXANE 1:1	0.76 ± 0.05	0.59 ± 0.01	$(0.1-0.15)^b$
3. ETHYL ACETATE- HEXANE 3:8	0.81 ± 0.02	0.56 ± 0.02	
4. ETHYL ACTATE- HEXANE 1:3	0.72 ± 0.02	0.30 ± 0.01	

NOTE: Each value represents 6 separate determinations. Except for systems 1 and 4 where $n=4$.

^aPublished value Ref. 151

^bPublished value Ref. 194

^cStandard deviation of mean - 6 determinations for each value

supernatant to 6 ml in the incubate resulted in an observable increase in the size of the shoulder past the Vitamin D peak (Fig. 16). However, there were still no observable differences in the various groups of animals tested, namely uremic, rachitic, as well as control rats. The shoulders in the chromatograms were found to be in the same region as was ^3H -25(OH) D_3 (Table VII) when it was co-chromatographed with the incubate extract.

Chrom-AR 1000 was used as a medium for thin layer chromatography to aid in the more rapid screening of possible metabolites during the evaluation of the various incubation systems (Table VIII). Here again, while the appearance of some hydroxy metabolite could be demonstrated by co-chromatography with authentic unlabeled 25-(OH) D_3 , it was still impossible to demonstrate any significant differences in most cases. In some cases a reduced appearance of radioactivity in the 25-(OH) D_3 region ($\text{rf} = 0.56$) could be observed (Table IX).

Additional investigations, using Sephadex LH-20 with chloroform-hexane in 1:1 (V:V) as a solvent system (112), gave a slightly better separation of calciferol and 25(OH) D_3 (Fig. 16). Re-chromatography of the 25(OH) D_3 region did improve the resolution of 25(OH) D_3 from Vitamin D, but still only a slight increase in metabolism of calciferol could be demonstrated in rachitic and uremic incubation systems (Fig. 17).

2. Metabolism of ^3H -(26,27)-25Hydroxy Vitamin D_3

In Vitro by Renal Homogenates

a. Rat renal homogenates

Initial studies, using rat renal homogenates to study the rate of hydroxylation of 25(OH) D_3 by renal mitochondria, proved to be inconsistent. Sephadex LH-20 chromatography of the extract obtained from the incubate demonstrated little evidence of metabolism. However, the appearance of increased activity in the

TABLE IX. REPRESENTATIVE CHROM-AR 1000 CHROMATOGRAMS OF ^{14}C -VITAMIN D_3 INCUBATED IN: (A) DENATURED LIVER HOMOGENATE, (B) CONTROL LIVER AND (C) UREMIC LIVER HOMOGENATE.

ETHYL ACETATE-n HEXANE (1:1, v:v) WAS USED TO DEVELOP THE STRIPS TO A SOLVENT FRONT OF 15cm. THE DEVELOPED CHROM-AR STRIPS WERE CUT INTO 1 cm PORTIONS AND RADIOACTIVITY DETERMINED.

Values are expressed as a percentile of total activity.

STRIP NUMBER	DENATURED INCUBATE	CONTROL	UREMIC
1	3.28	1.68	1.61
2	2.91	2.25	1.96
3	2.89	3.63	3.59
4	2.42	3.34	4.10
5	2.53	3.47	3.36
6	2.87	3.99	3.42
7	3.77	7.53	4.49
8	4.72	10.24	6.75
9	13.53	11.42	13.67
10	27.63	16.34	16.83
11	24.72	22.64	20.40
12	7.14	12.70	16.34
13	1.56	0.78	3.49
14	0.01	0.00	0.00
15	0.00	0.00	0.00
TOTAL ACTIVITY (DPM)	82.700	48.800	58.450

Representative of 5 control, 5 uremic and 5 rachitic rats

region where the dihydroxy-calciferols should occur was frequently observed when using the Chrom-AR 1000 chromatography system (Table X). The region of the Chrom-AR in which the increased activity occurred also had a lot of heavy lipids present, which may in fact have trapped the fat soluble ^3H -25(OH) D_3 and given rise to an artifact.

b. Avian Studies

To determine whether there was a problem in technique or methodology which prevented metabolism in rat kidney homogenate, chick kidney homogenate was used as a test system. It is well established that chick renal mitochondria metabolize 25(OH) D_3 very readily to 24,25(OH) $_2\text{D}_3$ or 1,25(OH) $_2\text{D}_3$, depending on the nutritional status of the donor (150-153). Using chick kidney homogenate to which had been added the necessary cofactors, plus 10 μl of ethanolic ^3H -25(OH) D_3 , it was possible to demonstrate conversion of a significant quantity of the substrate. In fact, more than 40% of the entire radioactivity of the extract was in the prime metabolite, 24,25(OH) $_2\text{D}_3$ (Fig. 19, Table XI). In the first series of experiments, it was also possible to demonstrate some 1,25(OH) $_2\text{D}_3$ production in the 4 week old chick kidneys. Studies at five and five and one half weeks no longer could demonstrate the presence of 1,25(OH) $_2\text{D}_3$, but the rate of production of 24,25(OH) $_2\text{D}_3$ had not diminished.

c. Repeated Studies of In Vitro Metabolism of 25-Hydroxy-Vitamin D_3 by Rat Renal Homogenates

By means of incubation conditions identical to those described for the avian system, the metabolic capacity of the control rat kidney was re-examined. It was found that significant metabolism could be demonstrated (Fig. 20). It was furthermore observed that doubling the quantity of NADP to the incubation mixture resulted in an increased metabolic conversion of 25(OH) D_3 to 24,25(OH) $_2\text{D}_3$, going from an initial rate of 14.4% to 25.6% (Table XI). Repeating these studies in rat kidney homogenates, obtained from uremic rats,

TABLE X. REPRESENTATIVE CHROMATOGRAMS OF EXTRACTS
OF ^3H -25(OH) D_3 RAT RENAL INCUBATES USING
CHROM-AR 1000 AS THE SEPARATING MEDIUM

STRIP NO.	A	B	C	D	E
0	0.21	2.69	3.03	2.62	1.50
1	0.18	4.38	2.53	2.84	1.23
2	0.36	9.81*	5.08*	5.06	1.10
3	0.43	1.09	3.75	8.14*	0.42
4	0.43	2.05	2.77	4.37	0.70
5	0.33	1.09	2.46	3.96	1.21
6	0.53	1.55	1.70	3.86	2.88*
7	0.72	1.73	1.86	3.02	1.66
8	0.92	1.50	1.51	2.97	1.34
9	10.33	12.51	5.69	15.69	1.47
10	60.85	36.43	37.06	34.85	2.30
11	22.67	22.10	27.72	11.65	3.07
12	1.10	2.73	4.59	0.61	10.57
13	0.24	0.22	0.34	0.03	35.25
14	0.0	0.0	0.0	0.00	28.84
15	0.0	0.0	0.0	0.0	6.08
16	0.0	0.0	0.0	0.0	0.25
17	0.0	0.0	0.0	0.0	0.0
TOTAL ACTIVITY (DPM)	72,200	48,700	58,500	87,300	34,700

Representative of 10 animals in each group.

The strips were extracted with 10 ml Chloroform-diethyl ether-ethanol (1:1:1, v:v), solvent evaporated and samples were then counted by means of liquid scintillation spectrometry. Solvent front = 17 cm. Strip number 0 = origin.

^a extract of substrate ^3H -25(OH) D_3 from denatured renal homogenate

^b control ^c rachitic

^d uremic incubate extract using ethyl acetate-n-hexane (1:1, v:v) as solvent

^e uremic renal incubate extract using 100% ethyl acetate

* Area in chromatogram suggestive of dihydroxy-Vitamin D_3 metabolites.

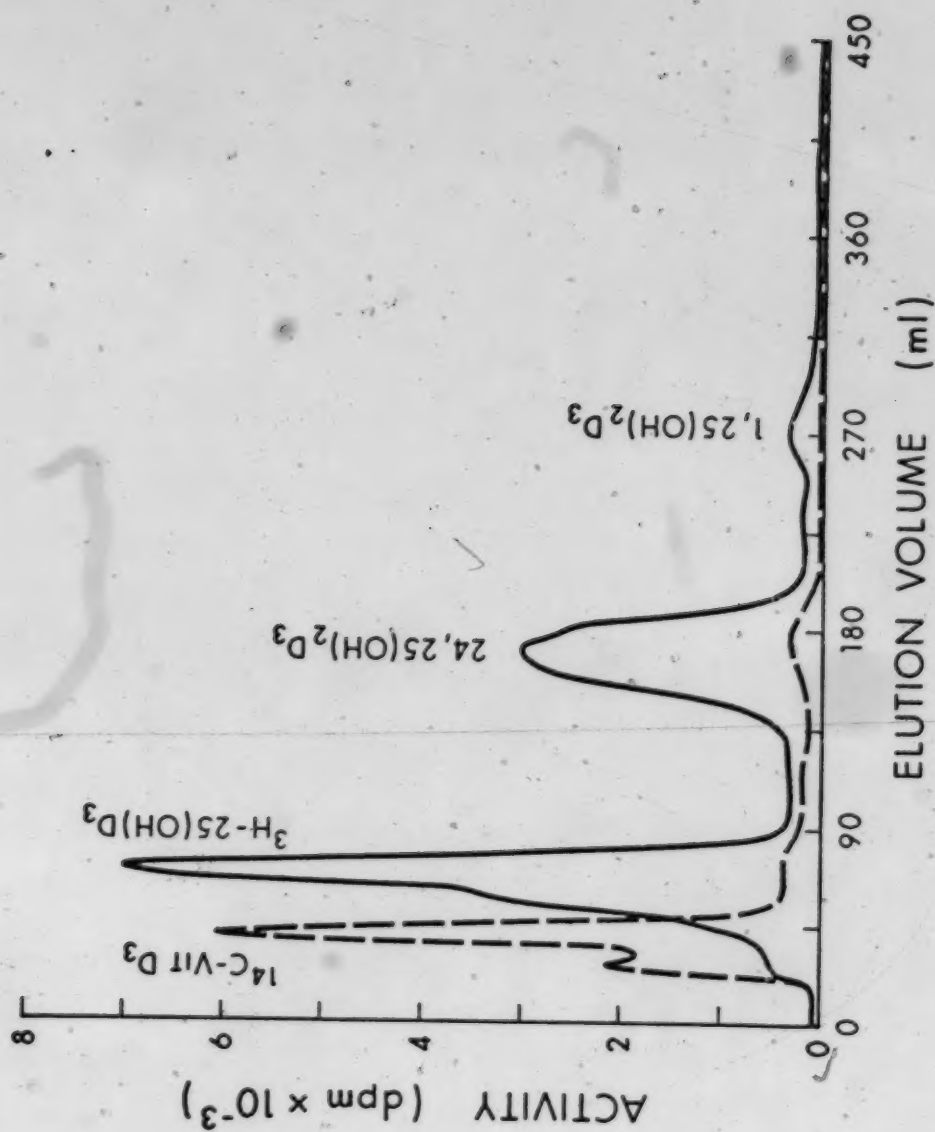


Figure 19: Sephadex LH-20 (1.3 x 50 cm column CHCl_3 -n-hexane 65:35, v:v) chromatographic profile of extracts of 25% chick renal homogenate (2 ml) incubated with ^3H -(26,27)-25 OHD_3 for one hour under an atmosphere of oxygen.

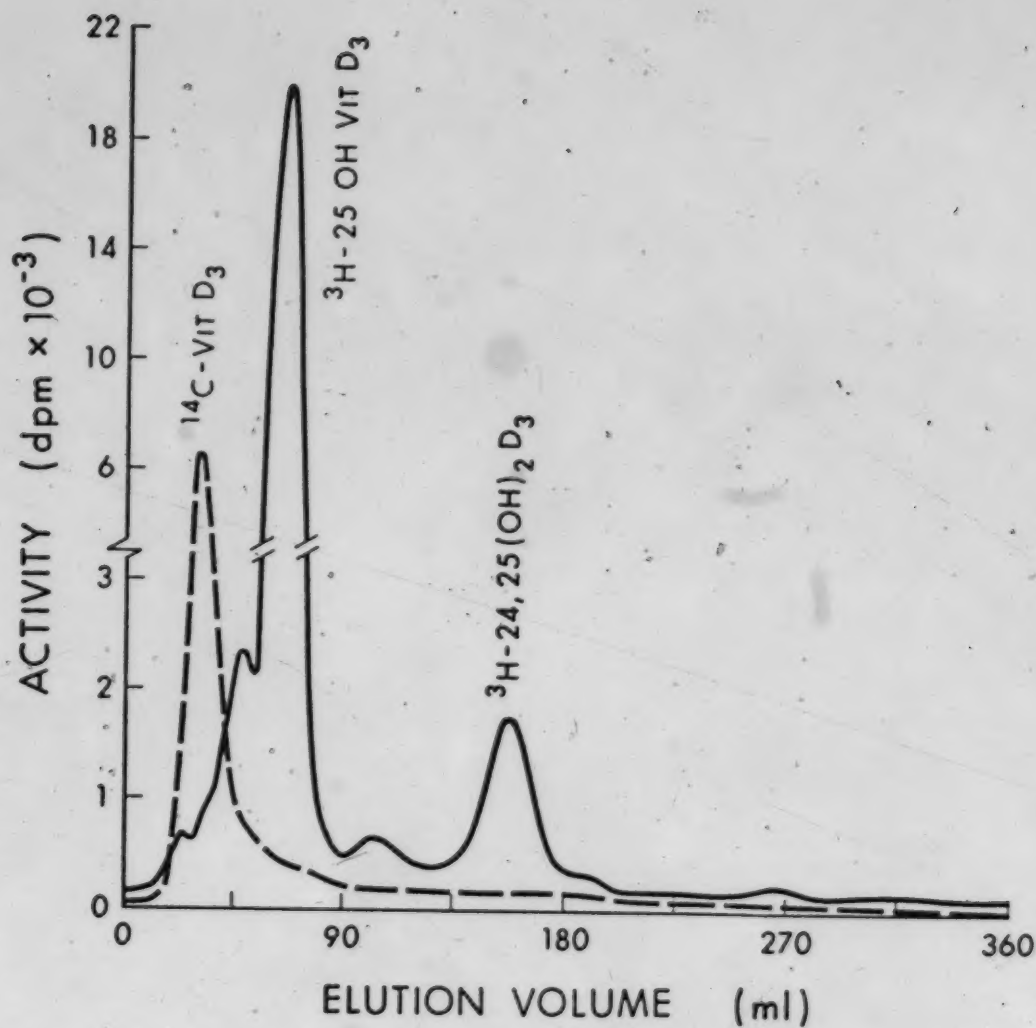


Figure 20: Sephadex LH-20 (1.3 x 50 cm column CHCl₃-n-hexane 65:35) chromatographic profile of extracts of 25% rat renal homogenate (2 ml) incubated with ³H-(26,27)-25(OH)D₃ for one hour under an atmosphere of oxygen.

TABLE XI. IN VITRO INCUBATION OF ^3H -25-HYDROXYVITAMIN D_3
 BY CHICK AND RAT RENAL HOMOGENATES:
 DISTRIBUTION OF METABOLITES AFTER SEPHADEX LH-20
 SEPHADEX LH-20 CHROMATOGRAPHY - EXPRESSED AS % OF TOTAL
 RADIOACTIVITY

HOMOGENATE USED	METABOLITES	
	$24,25(\text{OH})_2\text{D}_3$	$1,25(\text{OH})_2\text{D}_3$
CHICK (NORMAL DIET)	41.6 ± 2.2^a	3.82^b
RAT - CONTROL	14.4 ± 1.3^c	TRACE
	25.6^d	
RAT - UREMIC	$2.2-6.9^e$	--
	2.0^f	--

^a mean $\pm$ standard deviation of 8 chicks

^b mean of two chicks

^c mean of three animal in duplicate

^d mean of two animals using a twofold concentration of NADP (2μ moles)

^e range of three animals - (duplicate) - lower value pooled of two animals

^f value obtained from pooled kidney tissue of two animals using twofold NADP (2 moles)

demonstrated that in uremia there is a significant reduction in the hydroxylation activity of the renal mitochondria (Table XI). The quantity of ^3H -24,25(OH) $_2\text{D}_3$ produced was only 2 - 7% and the enzyme system failed to respond to the increased concentration of the cofactor NADP.

E. Effect of Uremia, and Rachetogenic Diet, on Hepatic Microsomal Enzyme Activity

1. Chronic Uremia (Table XII).

Chronic uremia was found to exert a depressing effect on the activity of hepatic microsomal enzymes. A significant reduction was observed in hexobarbital oxidase activity; having been reduced to 42.8% of normal. Aminopyrine demethylase activity was reduced to 46.3% of normal, while total p-nitrobenzoic acid reductase activity was reduced to 77% of normal. There was also a very significant concurrent reduction of cytochrome P-450 activity (56.8%). However, the microsomal protein was only slightly reduced.

2. Acute Uremia (Table XII).

Acute uremia induced by simultaneous sub-total and contralateral nephrectomy also had a very significant effect on microsomal enzymes. The hexobarbital oxidase activity was reduced to 61% of the control values, while the aminopyrine demethylase activity was found to be halved. The decrease in the total p-nitrobenzoic acid reductase was similar to the level observed in chronic uremic animals, while the cytochrome P-450 activity was 76% of normal. Microsomal protein again was found to be reduced, but only to a very small degree.

3. Effect of the Rachitic Diet (Table XII).

Maintaining animals on an artificial Vitamin D deficient diet for a prolonged period of time reduced the activity of the various hepatic microsomal enzymes. Oxidation of hexobarbital was reduced to 67%, aminopyrine demethylase to 60%, while the p-nitroreductase

TABLE XII. EFFECTS OF UREMIA, AND VITAMIN D DEFICIENT DIET ON HEPATIC
MICROSOMAL ENZYMES

TREATMENT	HEXOBARBITAL OXIDASE	AMINOPYRINE N-DEMETHYLASE	P-NITROBENZOIC ACID REDUCTASE p-AMINOBENZOIC ACID FREE	P-NITROBENZOIC ACID REDUCTASE TOTAL	CYTOCHROME P450 ^a	MICROSOMAL PROTEIN ^b
CONTROL vs CHRONIC UREMIA	5.36±0.19 2.29±0.15	0.92 ±0.06 0.43 ±0.02	0.31±0.04 0.25±0.02	0.53±0.04 0.41±0.02	30.45±1.45 17.60±1.62	21.36±0.56 19.48±0.78
CONTROL vs ONE STAGE UREMIC	5.55±0.15 3.39±0.18	0.91 ±0.03 0.45 ±0.06	0.32±0.02 0.24±0.02	0.54±0.03 0.42±0.01	31.70±1.12 24.20±1.90	21.76±0.56 19.17±0.99
CONTROL vs RACHITIC	5.34±0.33 3.61±0.33	1.00±0.08 0.59±0.08	0.34±0.03 0.20±0.01	0.52±0.01 0.29±0.01	31.70±1.02 25.85±0.78	21.49±0.57 20.69±1.42
CONTROL vs RACHITIC UREMIC	5.75±0.09 4.00±0.23	0.95 ±0.01 0.36 ±0.02	0.29±0.02 0.12±0.02	0.51±0.03 0.21±0.01	30.20±1.48 17.67±2.15	22.25±0.65 13.17±0.38

Activity of Hepatic Microsomal Enzymes expressed as μ moles metabolite formed per gram of liver (25% homogenate) $\pm$ s.e. per 60 min for hexobarbital oxidase and aminopyrine N-demethylase, while p-nitrobenzoic acid reductase is expressed per 30 minutes.

All values represent the mean values of 6 rats with the exception of chronic uremia where 10 rats were used.

a. m μ moles/g of liver

b. mg/g of liver

activity was reduced to 55%. Surprisingly, the cytochrome P-450 activity was only diminished to 82% of normal, and there was no significant effect on the microsomal protein level.

4. Combined Effect of Rachetogenic Diet and Chronic Renal Failure (Table XII).

The maintenance of uremic animals on a rachetogenic diet for a prolonged period of time appeared to have a devastating effect on hepatic microsomal enzyme activity. Aminopyrine demethylase and p-nitroreductase enzyme activities were observed to be less than half of normal activity. Hexobarbital oxidation was reduced to only 70% of normal, while cytochrome P-450 was 60% of control value. Microsomal protein levels were greatly reduced in the animals.

F. Glucocorticoid Levels in Acute, Chronic, Rachitic, Uremic and Control Rats.

Competitive protein binding assays of plasma from acute, as well as chronic uremic rats, and rachitic and uremic rachitic rats demonstrated that the circulating levels of corticosterone varied in each group of animals. The chronic uremic rats had a circulating levels three orders of magnitude higher than controls ($36.3 \mu\text{g}/100 \text{ ml}$ versus $11.4 \mu\text{g}/100 \text{ ml}$), while the acute uremic rats have been shown to have circulating levels which were five fold those of normal control animals ($51.3 \mu\text{g}/100 \text{ ml}$). Plasma corticoid levels in rachitic animals were found to be similar to those in control animals, while the rachitic uremic rats had circulating levels that were twice that of control animals (Table XIII).

G. Serum Calcium Levels in Acute, Chronic, Rachitic, Uremic, Rachitic and Control Rats

The results of the serum calcium determinations for the various groups (Table XIII) were not really surprising. The levels of the serum calcium in chronic uremia signified lower values than controls ($7.9 \text{ mg}\%$

TABLE XIII. THE EFFECTS OF UREMIA AND VITAMIN D₃ DEFICIENT DIET ON SERUM CALCIUM AND PLASMA-CORTICOIDS

TREATMENT	SERUM CALCIUM ^b	PLASMA CORTICOSTERONE ^c	BUN ^b
CONTROL	9.8±0.2	11.4±0.8	33.0±1.8
CHRONIC UREMIC	7.9±0.2	36.4±3.9	202.8±15.7
ONE STAGE ^d UREMIC	8.6±0.1	51.3±3.0	188.0±12.8
RACHITIC	5.8±0.7	11.2±0.7	--
RACHITIC UREMIC	4.2±0.3	25.5±1.9	48.5±3.2

^a Values in the table represent the mean ± standard error of the mean of 10 control, 10 uremic and 6 of each of the other groups performed in duplicate

^b Values expressed as mg/100 ml plasma

^c Values expressed as µg/100 ml plasma

^d Acute uremic 6 days post op after simultaneous subtotal and contralateral nephrectomy

versus 9.8 mg%). The one stage acute uremic rats demonstrated a slightly lower serum calcium level, but the value of 8.6 mg% is still within normal limits. As expected the animals maintained on a rachetogenic diet exhibited low values (5.8 mg%). The combination of chronic uremia plus maintenance on a rachetogenic diet proved to be quite a formidable challenge, resulting in serum calcium values of 4.2 mg%.

V. DISCUSSION

The objective of this research was to examine some of the biochemical parameters, specifically the activity of hepatic and renal enzymes, which may be altered as a result of pathophysiological changes which occur during the sequence of events leading up to the uremic syndrome.

In order to facilitate these studies, we chose to use the rat as a pathophysiological model for uremia, mainly because this species has been extensively used as a laboratory animal. It is easy to maintain, and it is quite resilient when exposed to various experimental situations, including surgical procedures.

A. Preliminary Evaluation of the Rat as a Pathophysiological Model for Uremia.

Upon examining the results obtained from the rats in which various surgical procedures had been used to induce uremia (Tables I and II), it became obvious that the procedure involving subtotal nephrectomy, followed a week later by contralateral nephrectomy, was far superior to the other procedures in achieving a persistent elevation of the BUN level. Furthermore, with this procedure there was a significant reduction in weight gain, such that 16 weeks post-operatively, the mean weight of these uremic animals was only 60% of the control value. On the other hand, the other procedures had little effect on weight gain or in the maintenance of persistent, elevated BUN levels over a prolonged period of time. Another useful finding was that the survival rate as a result of subtotal nephrectomy was better than 90%, over a four to six week period. During this time, most of the animals went through a state of transition which was marked by relative reduction of weight gain or even weight loss. This appeared to coincide with the renal compensation which occurred during this time period (209). These changes are supported by the data shown in figures 4 and 5, which depict compensatory changes in both the

glomeruli and convoluted tubules. The compensatory diuresis which had developed in these uremic animals would also be compatible with the observed doubling of the water consumption, when compared to the controls. Another feature observed in these uremic rats was impaired hemostasis, especially in the later stages of the disease. This had been demonstrated to be due to an inhibition of ADP-induced platelet factor 3 deficiency, in uremic patients (96); however, it has been shown to be reversed by dialysis (214).

Osteomalacia, a severe problem in uremic patients, could also be demonstrated to some extent in the uremic animals. Figure 3 indicates a reduced growth of the femur and possible reduced radio-density. The finding that the subtotally nephrectomized animals had a significantly reduced uptake of ^{45}Ca into long bones, can also be interpreted as signifying the presence of osteopenia or a disorder in mineral metabolism. The observation that long bones in the uremic rat appear to be dormant also implies the presence of abnormal bone mineral metabolism.

Histological examination of the liver (Fig. 6-11) also revealed that changes related to metabolism were occurring in the uremic liver; this was suggested by the increase in glycogen rosettes and increase in the quantity of rough endoplasmic reticulum.

In summary, the above findings would appear to indicate that the subtotally nephrectomized rat could serve as a useful pathophysiological model for uremia, in order to help elucidate some of the biochemical changes which occur in the chronic uremic syndrome.

B. Effect of Uremia on In Vivo Metabolism of ^{14}C -Vitamin D_3
 After an intraperitoneal injection of $0.5 \mu\text{Ci } ^{14}\text{C}$ -Vitamin D_3 (240 I. U.) into control and uremic animals, the blood levels of radio-activity representing ^{14}C -Vitamin D_3 and its metabolites were significantly

different in these two groups, as is indicated in Fig. 12. The more rapid appearance of radioactivity in uremic plasma would suggest a more rapid metabolic turnover of the injected ^{14}C -Vitamin D_3 , when compared to turnover in control animals. Furthermore, it suggests that there is a significant change in the endogenous pool of Vitamin D, as well as in storage sites in the uremic rat. Since the exogenous Vitamin D_3 , upon injection enters the endogenous Vitamin D pool and mixes with it, the ^{14}C -Vitamin D_3 and its possible metabolites would have to compete with unlabeled Vitamin D and metabolites for active transport sites on the plasma proteins, specifically the α globulin fraction (117, 211, 216). Since the total radioactivity in uremic blood was threefold that of control animals, this would suggest that in the uremic state, there is either a reduced endogenous pool of Vitamin D_3 and hence more plasma protein binding sites are available for combination with the radioactive Vitamin D_3 and its metabolites, or alternatively, more binding sites could be available in uremic plasma, suggesting there is a significant change in the albumin globulin ratio in uremic plasma. Since the α_1 globulin fraction (which is the carrier protein of Vitamin D_3 , 211) of plasma protein represents only 5-8% (210) of the total plasma proteins in normal individuals, even with proteinuria which occurs in uremia (213), it would still be unlikely that the α globulin fraction would increase by a magnitude of 3 in the uremic condition even though an abnormal protein synthesis has been demonstrated (57, 58). Because all animals were maintained on a normal diet, the changes observed should not be due to a dietary deficiency of Vitamin D_3 . Mawer *et al.* (115) demonstrated that the state of Vitamin D nutrition plays a significant role in metabolism of Vitamin D. The rate of metabolism was shown to be inversely proportional to the size of the endogenous Vitamin D pool.

The tissue distribution (Table IV) of radioactivity, representing ^{14}C -Vitamin D_3 and metabolites in uremic versus control tissue, also

supports the idea of the reduced endogenous Vitamin D pool in uremia, since total radioactivity in uremic liver is greater than two fold when compared to control liver (6830 dpm/g for uremic versus 2720 dpm/g for control livers). Similarly, the radioactivity was significantly higher as well, in the small intestine (3970 dpm/g for uremic versus 2260 dpm/g for controls) and kidney (14,500 dpm/g for uremics versus 11,300 dpm/g for controls). Data from Fig 13 demonstrates that in uremia, there occurs a more rapid metabolism of ^{14}C -Vitamin D_3 , since three metabolic peaks more polar than $25(\text{OH})\text{D}_3$ are very evident. These have been tentatively identified in $24,25(\text{OH})_2\text{D}_3$, peak Vb and $1,25(\text{OH})_2\text{D}_3$ (Table VII). In contrast, there is little evidence for the occurrence of peaks more polar than $25(\text{OH})\text{D}_3$ in control plasma (Table III). This, then, would suggest a lower rate of metabolism of the administered ^{14}C -Vitamin D_3 .

It is also interesting to note that in a group of rats which served as surgical controls, having had one kidney removed, the rate of metabolism was significantly increased, as is signified by the appearance of appreciable quantities of metabolites more polar than $25(\text{OH})\text{D}_3$ (Fig. 13c, Table III). While the number of dpm's in the dihydroxy metabolic peaks was smaller in the unilaterally nephrectomized rats compared to controls, the relative activity of the peaks is similar in both cases (Table III). This suggests that the size of the functional renal mass may play a significant role in affecting the rate of metabolic conversion of Vitamin D_3 . This, in part, is the basis for the Bricker hypothesis (212), that as the nephrons are removed from function, the load for reabsorbing phosphate is increased in the remaining nephrons. This in turn would result in reduction of the level of synthesis of $1,25(\text{OH})_2\text{D}_3$.

It should be noted that the values for the data in Table III and the graphs in Fig. 13 are from pooled plasma of each of three animals

in each group. While this is not statistically desirable, it was necessary because it was impossible to observe any dihydroxycalciferol peaks in individually chromatographed samples (Table III, lower part). Each value in this case is a mean of three separate determinations.

C. Effect of Uremia and Vitamin D Deficiency on the Metabolism of a Single Pulse Dose of ^{14}C -Vitamin D_3 and ^3H -25-Hydroxy Vitamin D_3

The pulse dose of combined ^{14}C -Vitamin D_3 and ^3H -25(OH) D_3 (0.25 μCi of each) was administered intravenously to rachitic, uremic and control rats in order to assess the metabolic performance in vivo of the liver and kidneys, particularly in the rachitic and uremic animals. The graphs in Fig. 14 signify the distribution of ^{14}C -Vitamin D and metabolites, and ^3H -25(OH) D_3 and metabolites in the blood of control, rachitic and uremic rats over a period of eight hours after intravenous injection.

It has been shown that both Vitamin D and its metabolites, in blood, are bound to plasma proteins (44, 215). Furthermore, the existence of a specific plasma transport protein in rat, chick and human plasma has been demonstrated (117, 211, 216). It has also been pointed out (217) that the affinity of 25(OH) D_3 is 100 times that of Vitamin D_3 for the specific binding site. For optimal binding, the 25^{OH} group was required as in the 5,6-cis configuration of the A ring of Vitamin D. Hydroxylation of the A ring in the carbon one position reduced the affinity for the binding site (211).

This information is of value in the interpretation of the data from graphs of control, rachitic and uremic rats. The ^3H -curve in control plasma can be interpreted as representative of the biological degradation of ^3H -25(OH) D_3 in vivo. The initial drop in the first 40 minutes could be interpreted as a period of equilibration between the ^3H -25(OH) D_3 and the endogenously plasma bound 25(OH) D_3 . The

rapid fall of the ^{14}C - blood levels can be interpreted as the uptake of ^{14}C -Vitamin D_3 by the storage compartments in the body. The plateau can be taken to represent both some bound ^{14}C -Vitamin D and to a larger extent, newly synthesized ^{14}C - $25(\text{OH})\text{D}_3$.

Upon examination of the levels of activity of ^3H and ^{14}C , it can be seen that the pattern of biological decay of the compounds is markedly different. The initial slope of the ^3H blood activity over the first 160 minutes could be interpreted as representing the rate of biological degradation (metabolism) of ^3H - $25(\text{OH})\text{D}_3$. As can be observed, the slope over the first 160 minutes is much steeper than that of the control ^3H blood levels. This can be accounted for by the more rapid metabolism of ^3H - $25(\text{OH})\text{D}_3$ to the dihydroxy metabolites, especially $1,25(\text{OH})_2\text{D}_3$, and is supported (Table V) by the increased appearance of $1,25(\text{OH})_2\text{D}_3$ in rachitic plasma when compared to controls. The steeper slope would therefore suggest a shortened biological half-life of $25(\text{OH})\text{D}_3$ in Vitamin D_3 deficient animals, which is to be expected. The increase in tritium activity and consequent reduction of the slope past 160 minutes, in the rachitic animals, can be interpreted as the effect of release of ^{14}C -labeled $25(\text{OH})\text{D}_3$ by the liver into the blood stream. This hypothesis is supported by the observed rise in ^{14}C blood radioactivity at this time, followed by a steady increase in the slope of the curve representing ^{14}C blood activity. Table IV depicts the distribution of plasma metabolites of Vitamin D eight hours after intravenous injection; this data signifies that in rachitic rats the ^{14}C - $25(\text{OH})\text{D}_3$ represents 21.2% of the total plasma activity compared to 11.9% found in control rats. This supports the expectation that in rachitic rats, the liver metabolizes the injected ^{14}C -Vitamin D_3 more readily, due to the obvious reduction in the endogenous Vitamin D pool in these animals. Therefore, the rise in ^{14}C blood levels in rachitic rats can be seen to represent the rate of synthesis of ^{14}C - $25(\text{OH})\text{D}_3$ and the subsequent metabolism of this compound to the dihydroxy metabolites.

Upon examination of the graph representing the blood ^3H and ^{14}C levels in uremic animals, one can observe the sharp decline of plasma ^3H activity during the first 60 minutes after intravenous injection, followed by a leveling of the plasma activity. This plateauing coincides with the rapid rise in ^{14}C blood activity. The initial sharp drop in the ^{14}C blood activity can suggest the rapid uptake of ^{14}C -Vitamin D_3 by the liver. The sudden rise and subsequent fall in activity between 30 and 90 minutes is suggestive of the release of ^{14}C -25(OH) D_3 by the liver as well as the continued uptake of ^{14}C -Vitamin D_3 by the same organ (218). Then, at 90 minutes, the release of ^{14}C -25(OH) D_3 by the liver exceeds the uptake of the remaining ^{14}C -Vitamin D_3 , hence giving rise to the steady increase in the slope over the 8 hour test period. The ^{14}C -25(OH) D_3 would therefore enter the ^3H -25(OH) D_3 pool that is bound to the plasma protein, hence competing with ^3H -25(OH) D_3 for metabolism. This can be seen in the steady level of ^3H in blood from 280 minutes to the end of the eight hours of the test. This region corresponds to the increase in the slope of ^{14}C -25(OH) D_3 . Unfortunately, the data on Table V does not represent the same animals as does the graph in Fig. 14. The data presented in Table V represents a repeat experiment of 3 uremic animals and the rate of biotransformation of ^{14}C -Vitamin D_3 and ^3H -25(OH) D_3 is greatly reduced in these animals.

The plasma distribution of metabolites (Table V) in control animals was as one would expect in normo-calcemic individuals. The conversion of ^{14}C -Vitamin D_3 was similar (11.9% of total plasma activity) to the value obtained in the initial experiment (Table III), where ^{14}C -25(OH) D_3 represented 10.6% of the total activity. The pooling of plasma obtained from 6 control rats allowed the visualization of the dihydroxy metabolites of Vitamin D_3 . It can also be demonstrated that the principal metabolite of ^3H -25(OH) D_3 is 24,25(OH) $_2\text{D}_3$ which is to be expected in normo-calcemic individuals (150).

In rachitic plasma, the ^{14}C -25(OH) D_3 formed a significantly larger percentage (21.2%) of the total plasma ^{14}C -radioactivity when compared to the control levels, which is not surprising, since reduced endogenous Vitamin D pools have an accelerating effect on the rate of biotransformation of Vitamin D_3 (115). The slightly larger observed quantity of ^{14}C -24,25(OH) $_2\text{D}_3$ (1.7%), compared to ^{14}C -1,25(OH) $_2\text{D}_3$ (1.4%), may not be significantly different or this may be due to the inhibiting effect that ^3H -25(OH) D_3 may have, since it is formed directly from the administered ^3H -25(OH) D_3 while the ^{14}C -1,25(OH) $_2\text{D}_3$ is derived from ^{14}C -25(OH) D_3 which first has to be synthesized in the liver from the administered ^{14}C -Vitamin D_3 . Therefore, by expressing its physiological action, the ^3H -1,25(OH) $_2\text{D}_3$ can significantly alter the calcium milieu so as to affect the rate of synthesis of ^{14}C -1,25(OH) $_2\text{D}_3$ to some extent. The ^3H -25(OH) D_3 certainly expresses its physiological effects through 1,25(OH) $_2\text{D}_3$ synthesis by the kidney, within 4-6 hours after injection (158).

The synthesis of the 1,25(OH) $_2\text{D}_3$ is reduced in the uremic rats to trace levels, while greater 24,25(OH) $_2\text{D}_3$ levels can be observed. These findings are also similar to those in Table III, where in uremic plasma, a slightly larger quantity of 24,25(OH) $_2\text{D}_3$ (5.3%) could be observed compared to 1,25(OH) $_2\text{D}_3$ (4.1%), and would be in keeping with Bricker's hypothesis (212).

The tissue distribution data for ^{14}C -Vitamin D_3 and its metabolites, and for ^3H -25(OH) D_3 and its metabolites is expressed in Table VI. There appears to be no significant difference in the amount of ^{14}C activity or tritium activity observed in all three groups. While the kidney tissue is not a storage depot per se for calciferol, one would expect both uremic and rachitic tissues to have higher concentrations of radioactivity. In the liver, there is a significant increase in radioactivity of Carbon 14, representing Vitamin D and, to a lesser extent, the metabolites. However, no significant difference exists between the radioactivity levels in uremic and control livers. These results are not in agreement

with those shown in Table III. Different routes of administering the hormones were used; in the first case, the intraperitoneal route was used and in the second instance, the intravenous route was used. It is not likely that the route of administration should have such a significant effect, at least not in this case, since we are dealing with lipid soluble substances which easily diffuse through biological membranes.

Different methods were also used in obtaining the two sets of data. The data in Table III was arrived at by extracting the tissues by the method of Blight and Dyer, scintillation counting an aliquot and chromatographing the remaining material on Sephadex LH-20 column. Subsequently, the activity in each of the collected fractions was determined by means of liquid scintillation spectrometry, taking care to correct the sample results for changes in counting efficiency. The method of Blight and Dyer has been shown, over the years, to be an efficient procedure for the extraction of fat soluble materials. Extraction efficiency was found to be 95-99%.

The data for Table VI was obtained by means of oxidizing the tissues in a Packard 306 oxidizer. This is a very rapid procedure for determining tritium or carbon-14 activity since the tritium is oxidized to ${}^3\text{H}_2$ and the ${}^{14}\text{C}$ to ${}^{14}\text{CO}_2$. Both are trapped and the activity can easily be determined. Standards of ${}^3\text{H}$ and ${}^{14}\text{C}$ -hexadecane were used to monitor the rate of recovery. The recovery values suggested that the oxidizer operated at a high level of efficiency. Therefore the differing procedures, in all likelihood, would not play a significant role in explaining these differing results. It may well be that the "one pass" effect in the intraperitoneally injected animals did play a significant effect, while in the intravenously injected animals, the storage depots were subjected to a high pulse dose of radioactivity. The lipid soluble ${}^{14}\text{C}$ -Vitamin D_3 and ${}^3\text{H}$ - $25(\text{OH})_2\text{D}_3$ may have been distributed in all body tissues containing lipids, this includes liver and kidney, irrespective of the concentration of Vitamin D_3 already present.

D. In Vitro Metabolism of ^{14}C -Vitamin D_3 and ^3H -25-Hydroxy Vitamin D_3

1. Metabolism of ^{14}C -Vitamin D_3 In vitro

As previously mentioned, the results of the in vitro experiments proved to be disappointing, primarily because it was difficult to demonstrate any significant change in the rate of metabolic conversion of ^{14}C -Vitamin D_3 , under a variety of experimental conditions such as uremia or Vitamin D deficiency. Our initial objective had been to examine the changes that occur in hepatic hydroxylation of Vitamin D, since metabolic changes obviously do occur in vivo.

From the data in Figures 15 to 18 and Table IX, we have been able to demonstrate some metabolism of Vitamin D to its hydroxylated metabolite; the rate of biotransformation, however, is low. This finding is similar to that observed by Bhattacharyya and DeLuca (124, 125, 145, 146). Changing the elution solvent system to CHCl_3 -n-hexane (1:1, v:v) (112) appeared to improve the resolution of Vitamin D and $25(\text{OH})\text{D}_3$ on the column; (Fig. 17) and the quantitative results appeared to hint, at least, at small differences in the rates of metabolism. The rechromatography of the $25(\text{OH})\text{D}_3$ region (Fig. 18) did show some small differences. In controls, the smaller peak, $25(\text{OH})\text{D}_3$, made up 9.9% of the total activity, while the uremic incubate produced 10.6% and the rachitic a slightly higher level of 13.5%. These levels hardly reflect those found in vivo, especially in the rachitic animals (Table V). However, the control values for the rate of synthesis of ^{14}C - $25(\text{OH})\text{D}_3$ in the incubate are similar to the values found in vivo in control animals (Tables III and V). These in vivo values for $25(\text{OH})\text{D}_3$, however, reflect both the rate of formation of this metabolite and its further metabolism to the dihydroxy metabolites of Vitamin D_3 . It may well be that the incubation conditions were still not ideal to truly reflect the level of activity of the 25-hydroxylase enzyme, particularly in the case of the rachitic liver incubates. It is conceivable that by maintaining the animals on a rachetogenic diet for a period of

time (8-10 weeks), various endogenous co-factors in the cytochrome P-450 electron transport chain were reduced to such a level that the rate limiting factor in the enzyme activity was the quantity of co-factors added, and not the activity of the 25-hydroxylase enzyme (225).

2. Metabolism of ^3H -(26,27)-25-Hydroxy Vitamin D_3 In Vitro by Renal Homogenates

Initial studies of in vitro metabolism appeared to be inconsistent. Sephadex LH-20 chromatography provided little evidence for the appearance of significant quantities of metabolites. To provide more rapid screening of the incubates, Chrom AR 1000 was used (Table VIII). Data presented in Table X certainly would indicate that a significant biotransformation of ^3H -25(OH) D_3 to the dihydroxycholecalciferols had occurred. Column 'A' represents ^3H -25(OH) D_3 which had been extracted from denatured rat renal homogenate. Column 'B' is the same viable kidney homogenate which had been incubated with ^3H -25(OH) D_3 for one hour. One would suspect metabolism to have occurred. However, rechromatography of a large aliquot of the extract on a Sephadex LH-20 column demonstrated little evidence of a metabolite more polar than ^3H -25(OH) D_3 .

These perplexing findings caused us to reassess our experimental approach. By using chick kidney homogenates with Tris-acetate buffer instead of Tris-HCl buffer, we were able to demonstrate a very significant rate of hydroxylation (Table XI, Fig. 19). Repeating these experiments under the same conditions, using kidney homogenates from control rats, gave rise to a significant amount of metabolism (Fig. 20, Table XI). Gray *et al.* (220) have suggested that the acetate ion of the buffer is necessary for full enzyme activity. These results would support that idea. It was also found that increasing the NADP⁻ concentration in the incubation medium from 1 μ mole to 2 μ moles significantly increased the formation of 24,25(OH) $_2\text{D}_3$ in the rat renal homogenate preparation from 14% to 25.6% of the substrate. There appears to be some disagreement on the role of

extramitochondrial NADP. Gray *et al.* (220) and others (228) suggest that NADP supports mitochondrial hydroxylation by a possible transhydrogenase which is active in transferring electrons into mitochondria. Lehninger (229) supports this idea by demonstrating that electrons can and do enter mitochondria from the cytoplasm via the glycerol phosphate shuttle or malate shuttle. Norman and co-workers (158, 159) suggest its role is of no significance.

Repeated studies in a limited number of renal homogenates obtained from chronically uremic rats signified a reduction in the rate of synthesis of $24,25(\text{OH})_2\text{D}_3$ to 2-6.9% which is less than half of the normal activity of these enzymes (Table XI). Furthermore, the degree of uremia appears to have had an inverse effect on the rate of hydroxylation by the renal mitochondria. The more debilitated the animal appears, the lower the rate of synthesis of $^3\text{H}-24,25(\text{OH})_2\text{D}_3$ (2% versus 6.9% in a fairly "healthy" uremic rat). The severely uremic animals also did not respond to increases in the presence of NADP in the incubation mixture. It may well be that the uremic toxins either have some effect on the respiratory chain or have a direct inhibitory effect on the mitochondria hydroxylating system. In other systems, such as rat liver mitochondria, it has been shown that oxidative phosphorylation is inhibited by serum from uremic patients (71). However, it is also conceivable that the uremic renal mass contains fewer mitochondria per gram of wet weight of renal tissue, therefore effectively reducing the quantity of mitochondrial hydroxylating enzymes in the uremic renal homogenates.

E. The Effect of Uremia and Rachetogenic Diet on Hepatic Microsomal Enzymes

1. Chronic Uremia

Data in Table XII demonstrates that chronic uremia exerts a general depressing effect on the mixed function oxidase systems in the liver microsomes. The decrease of hexabarbitol oxidase to 42.8% of normal, aminopyrine demethylase to 46.3% and p-nitrobenzoic acid reductase to 77% of controls, parallels the reduction in the amount of cytochrome P-450 which is found to be reduced to 56% of control values.

Surprisingly, only a slight reduction of microsomal protein occurred. Protein synthesis, in the uremic liver may be greater than normal, as shown by the increase in RER (Figures 8-11) and the finding that amino acids are incorporated into protein at an accelerated rate (57, 58). However, our observation was that this protein did not seem to be directed at increasing oxidative enzymes in the liver endoplasmic reticulum. In addition, it has been shown that these animals have significant proteinuria (55). This would explain why the microsomal protein content of the liver did not parallel the reduction of cytochrome P-450 activity or other measured enzyme activity. The reduction in enzyme activity could also be interpreted, at least partially, as a direct toxic effect of the increased concentration of noxious nitrogenous substances. It has been demonstrated that ultrafiltrate from the serum of azotemic patients exerts an inhibitory effect on a number of enzyme systems in the guinea-pig brain slice preparation (72). There was significant reduction of activity in dopa decarboxylase, monoamine oxidase, lactic dehydrogenase and a number of other enzyme systems. It has been shown (63) that experimentally induced uremia in rats prolongs the sleeping times with the administration of thiopental, hexobarbital and a number of other barbiturates. Further, these workers (63,64) showed that elevation of blood non-protein nitrogen, by injection of a solution of urea, uric acid, creatinine and creatine, prolonged the sleeping time of rats. Parallel observations were made in azotemic patients (221).

Recently, Bhattacharyya and DeLuca (145) pointed out that hydroxylation of calciferol occurs in the hepatic microsomes, rather than the hepatic mitochondria, contrary to previous theories (124). It has been well established that hydroxylation of calciferol is reduced in the uremic state (44,45).

Another vital consideration in uremia is the stress of the azotemic state in itself. Data from Table XIII suggests that in the

chronic uremic state, there is an elevation of the circulating corticosteroids to a level three times that of the control animals. The elevation of serum glucocorticoids in the subtotally nephrectomized rats matches the increase of glucose-6-phosphate dehydrogenase activity which would lead to increased utilization of the monophosphate oxidative pathway (pentose shunt) (55), resulting in increased synthesis of lipids and lipoproteins.

It is a well accepted fact that stress and subsequent release of steroids can initiate hepatic microsomal enzyme induction (75-81). However, these steroids are metabolized by these same mixed function oxidases and can competitively inhibit microsomal oxidation of xenobiotics (82-85). This implies that stress alone or, more likely, a combination of stress and other complications of uremia can strongly influence microsomal enzyme activity. It is interesting to note that Shafrir (90), using a uremic model produced by injecting rats with aminonucleoside of puromycin, found that the blood corticoid levels were reduced in comparison to controls.

Our observations of reduction of hepatic microsomal mixed function oxidases were paralleled by those of Leber (222,223). They used an acute uremic model. Estimations of enzyme activities were performed six days after combined subtotal and contralateral nephrectomy. They found reductions of the following order: hexabarbitol oxidase - 63% of normal; aminopyrine-N-demethylase - 65% of normal; aniline hydroxylation - 50% of normal; and cytochrome P-450 - 68% of normal. In further studies (223), the same workers demonstrated a decrease in O-demethylation of p-nitroanisole (62%) and in acetanilide hydroxylation (69%). The general trend of their data agrees with our results except that our values for aminopyrine-N-demethylase and hexabarbitol oxidase were lower. This gives rise to a dichotomy of theories. What is actually being estimated, the effect of uremia on hepatic microsomal enzymes or the effect of post-operative stress combined with acute uremia and its effect on mixed function oxygenases?

In an attempt at clarification, we repeated the experiments to see if, in fact, the surgical stress does play a significant role in the activity of hepatic mixed function oxygenases in acutely uremic rats.

2. Acute Uremia

Using rats made uremic in a one stage operation, the activity of the hepatic microsomal mixed function oxygenases was determined and is recorded in Table XII and the effect on circulating corticosteroids is reported in Table VIII. One of the greatest obstacles in this experiment was to obtain six day survivors because the mortality rate was 65-75% over the six day period. These studies signified a reduction of activity of hexabarbitol oxidase to 61% of control value, while aminopyrine demethylase was reduced to 50% and p-nitrobenzoic acid reductase to 77%. It is interesting that there is no significant difference ($P \geq 0.05$) in the synthesis of p-amino benzoic acid both in the free form and total (free plus conjugated) metabolites between the acute and the uremic animals. Nor does either group of animals exhibit a change in the rate of conjugation of p-amino-benzoic acid. This is determined by subtracting the amount of 'free' p-aminobenzoic acid formed from the 'total' metabolite (representing free plus conjugated p-aminobenzoic acid). While there is a general reduction in p-nitroreductase activity, there is no change in the relative (percentage) amount of conjugation in either acute or chronic uremia. This is in agreement with the findings of Reidenberg (97), who suggested that generally, there was no change in the rate of conjugation in uremia.

The cytochrome P-450 activity was reduced to 76% of control values in acutely uremic rats. Microsomal protein was only slightly lower than in the controls. These values are somewhat higher than those obtained for the chronic uremic animals. Of particular interest are the plasma corticoid values (Table XIII) which are almost twice the chronic uremic levels and 4.5 times the control levels. This raises the question as to how significant a role these elevated steroid levels play in both enzyme induction and competitive inhibition, combined

with the toxic effect that occurs as a result of the nitrogenous waste accumulation (71-74). The serum calcium in these animals, while still within the lower normal limits, was nevertheless significantly reduced from the normal values. This suggests that either the higher steroid levels or the reduction of the renal mass or a combination of these factors, would alter the metabolic pattern of calciferol so significantly that little $1,25(\text{OH})_2\text{D}_3$ would be produced; this in turn, being reflected as lower serum calcium values. Alternatively, there could be a defect of calcium transport in the small intestine due to the uremic toxins (49) and/or steroids (36,38). From a physiological, as well as biological, point of view, the acute uremic animal is significantly different from the chronic uremic animal.

3. Effect of Rachitic Diet on Microsomal Metabolism

This portion of the study was undertaken to ascertain the role that the rachitic state plays in the activity of hepatic microsome mixed function oxygenases. Nutritional status has long been recognized as an important factor in hepatic metabolism of drugs (224).

The data in Table XII is certainly indicative that rats placed on a rachetogenic diet for eight weeks have a reduced microsomal enzyme activity. While the hexabarbitol activity and aminopyrine-N-demethylase is reduced, the largest reduction is in the p-nitrobenzoic acid reductase activity. Whereas in all the other animals, both uremic and control, the conjugated form of p-nitrobenzoic acid (the metabolite of p-nitrobenzoic acid) is in the order of 40% of the total p-amino-benzoic acid produced, in the rachitic rat, the conjugated form only represents 30% of the total metabolite. In contrast to these observations, the cytochrome P-450 is reduced to only 82% of normal, while there is no significant change in the microsomal protein. Change would signify that possibly various co-factors required in the electron transport chain are reduced (225,226) resulting in the reduction of the activity of microsomal enzymes. Other workers (225,226) have shown that endogenous cofactors such as glucose-6-phosphate are greatly reduced in

animals placed on semisynthetic diets for a prolonged period of time.

4. Combined Effect of Rachetogenic Diet and Chronic Renal Failure

The data in Tables XII and XIII demonstrate that these animals express a combined effect of chronic uremia and Vitamin D deficiency. The decrease of hexabarbitol oxidase to 69%, aminopyrine-N-demethylase to 37%, and total p-nitrobenzoic acid reductase to 41% parallels the reduction of cytochrome P-450 to 58% of control values, while the microsomal protein was reduced to 56% of normal. The elevated plasma corticoids would also exert their influence on the microsomal oxidase as suggested previously. Of great interest is the reduced BUN levels in these animals. They were just slightly above normal, compared to other uremic animals. The low BUN levels plus the greatly reduced microsomal protein would suggest that the rachetogenic diet does not supply the necessary protein requirement for normal physiological and biochemical activity. Great care should be taken when extrapolating data from animals which have been on a restricted synthetic diet such as the Rachetogenic Diet No. 2 (Nutritional Biochemical) for a prolonged period of time. Data obtained from animals on this diet certainly does not project the biochemical events which occur in animals on a regular diet.

VI. SUMMARY

1. The subtotally nephrectomized rat is a useful model for studying the effects of chronic uremia on various biochemical parameters. This model exhibits many of the clinical symptoms which are associated with chronic uremic patients.
2. These animals presented a persistent elevation of BUN, demonstrated a significant reduction in weight gain, increased water consumption and impaired hemostasis.
3. There was significant reduction in mineral uptake, especially calcium, in the uremic bone. The femur on x-ray examination appeared less radiodense, and upon histochemical examination, appeared to be dormant with respect to mineral deposition.
4. Changes in the uremic liver were observed, especially by means of electron microscopy, indicating increased quantities of RER as well as glycogen. In the uremic kidney, the glomerulus was greatly enlarged, as were the convoluted tubules.
5. After a group of uremic and control rats had been given an intraperitoneal injection of $0.5 \mu\text{Ci}$ (240 I. U.) ^{14}C -Vitamin D_3 , serial blood samples taken over a period of 24 hours, at specific time intervals, illustrated a more rapid increase in the blood radioactivity in uremic rats as compared to controls. After 6 to 8 hours, there was a three fold difference.
6. Examination of the plasma of control, uremic and unilaterally nephrectomized rats, which had been given an i. p. dose of $0.5 \mu\text{Ci}$ (240 I. U.) ^{14}C -Vitamin D_3 , by means of Sephadex LH-20 Chromatography, revealed the presence of significant quantities of metabolites more polar than $25(\text{OH})\text{D}_3$ in uremic animals. This was also observed in unilaterally nephrectomized (one kidney only) rats. In contrast, no significant quantities of these metabolites could be found in plasma obtained from control rats.
7. Tissue distribution of ^{14}C -Vitamin D_3 and metabolites in these animals revealed a significant increase of radioactivity in uremic liver, kidney and small intestine, on a per gram basis.

8. Control, uremic and rachitic rats were given an intravenous injection of 50 μ l ethanolic solution containing ^{14}C -Vitamin D_3 and ^3H -25(OH) D_3 (0.25 μCi of each); serial blood samples were taken over an 8 hour period for the determination of blood radioactivity. In control animals, the blood tritium levels representing circulating ^3H -25(OH) D_3 and metabolites declined slowly over a period of 8 hours, so that at 8 hours, 60% of the tritium activity still remained. In contrast, the carbon-14 blood activity, representing circulating ^{14}C -Vitamin D_3 and metabolites, dropped very sharply over the first 40 minutes, then maintained a level of 25% compared to initial levels.

In rachitic rats, the blood tritium levels declined somewhat more readily than controls, over the first 180 minutes post injection. Then the rate of decline was reduced; this coincided with a slight rise in Carbon-14 activity which occurred at this time. The carbon-14 levels declined rapidly over the first 40 minutes and then leveled off somewhat. One hundred and sixty minutes after injection, the blood carbon-14 activity increased slightly over the next five hours.

In uremic animals, the tritium activity declined somewhat rapidly over the first 60 minutes and then decreased at a lower rate. This rate of change of tritium activity in the blood was paralleled by a sharp increase of Carbon-14 activity at this time, which increased very markedly over the next 7 hours.

9. Examination of plasma chromatographic profiles from these groups of animals revealed that control animals metabolized significant quantities of ^{14}C -Vitamin D to 25(OH) D_3 and 24,25(OH) $_2\text{D}_3$. Similarly, ^3H -25(OH) D_3 is metabolized to 24,25(OH) $_2\text{D}_3$. Small quantities of ^{14}C and ^3H -1,25(OH) $_2\text{D}_3$ were also present. Rachitic rat plasma demonstrated a similar but exaggerated effect, with the exception that the prime dihydroxy metabolite produced was 1,25(OH) $_2\text{D}_3$. In contrast, uremic animals displayed a reduction in the quantities of dihydroxy metabolite. Only 24,25(OH) $_2\text{D}_3$ was observed.

10. Studies involving in vitro metabolism of ^{14}C -Vitamin D_3 revealed that while it was possible to demonstrate metabolism of ^{14}C -Vitamin D_3 to ^{14}C - $25(\text{OH})\text{D}_3$ it was impossible to demonstrate any significant difference in the rate of metabolism between control, rachitic and uremic rat liver preparations.
11. In vitro studies using normal chick kidney homogenates demonstrated 40% metabolism of the ^3H - $25(\text{OH})\text{D}_3$ substrate to ^3H - $24,25(\text{OH})_2\text{D}_3$. Normal rat kidney homogenate under similar conditions demonstrated metabolism of ^3H - $25(\text{OH})\text{D}_3$ to ^3H - $24,25(\text{OH})_2\text{D}_3$ at a rate of 14% after 1 hour incubation. Increasing the quantity of NADP in the incubate twofold resulted in an increase to 25% metabolism of ^3H - $25(\text{OH})\text{D}_3$.
- Uremic rat renal homogenates demonstrated a reduction of transformation of ^3H - $25(\text{OH})\text{D}_3$ from 14% to 2-7%. The uremic incubate did not appear to respond to increased concentrations of NADP in the incubation medium.
12. Uremia causes a reduction in the activity of hepatic mixed function oxygenases. This may be due to circulating uremic toxins. Also, elevated circulating corticosterone levels may play a significant role in affecting the activity of the microsomal enzymes.

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APPENDIX

TABLE VIX. NORMALIZED VALUES OF THE RADIOACTIVITY OF 100 μ l
BLOOD SAMPLES IN CONTROL, RACHITIC AND UREMIC RATS AFTER AN
INTRAVENOUS INJECTION OF 50 μ l ETHANOL, CONTAINING 0.25 μ Ci EACH
OF ^3H -25(OH) D_3 AND ^{14}C -VITAMIN D_3 .

Blood activity values are compared to the 5 min post-injection value;
each value represents mean $\pm$ S.D. of 6 animals.

a. Tritium Blood Activity

Time of Sample	Control	Rachitic	Uremic
5	1	1	1
10	1.04 $\pm$ 0.06	0.96 $\pm$ 0.21	0.94 $\pm$ 0.06
15	0.94 $\pm$ 0.14	0.90 $\pm$ 0.18	0.88 $\pm$ 0.09
20	0.91 $\pm$ 0.13	0.83 $\pm$ 0.17	0.90 $\pm$ 0.08
25	0.94 $\pm$ 0.13	0.91 $\pm$ 0.11	0.86 $\pm$ 0.10
30	0.92 $\pm$ 0.15	0.80 $\pm$ 0.14	0.87 $\pm$ 0.06
40	0.86 $\pm$ 0.11	0.90 $\pm$ 0.13	0.84 $\pm$ 0.10
50	0.97 $\pm$ 0.04	0.84 $\pm$ 0.22	0.77 $\pm$ 0.13
60	0.92 $\pm$ 0.05	0.86 $\pm$ 0.22	0.78 $\pm$ 0.12
70	0.93 $\pm$ 0.12	0.82 $\pm$ 0.19	0.78 $\pm$ 0.12
80	0.93 $\pm$ 0.08	0.91 $\pm$ 0.11	0.75 $\pm$ 0.12
90	0.93 $\pm$ 0.06	0.79 $\pm$ 0.10	0.76 $\pm$ 0.10
100	0.88 $\pm$ 0.10	0.78 $\pm$ 0.17	0.73 $\pm$ 0.06
110	0.86 $\pm$ 0.08	0.72 $\pm$ 0.22	0.72 $\pm$ 0.15
120	0.83 $\pm$ 0.13	0.74 $\pm$ 0.11	0.70 $\pm$ 0.08
140	0.85 $\pm$ 0.07	0.77 $\pm$ 0.09	0.70 $\pm$ 0.06
160	0.78 $\pm$ 0.07	0.69 $\pm$ 0.16	0.71 $\pm$ 0.07
180	0.80 $\pm$ 0.09	0.70 $\pm$ 0.09	0.68 $\pm$ 0.07
200	0.81 $\pm$ 0.09	0.71 $\pm$ 0.10	0.68 $\pm$ 0.05
220	0.80 $\pm$ 0.07	0.70 $\pm$ 0.11	0.67 $\pm$ 0.09
240	0.76 $\pm$ 0.06	0.71 $\pm$ 0.13	0.60 $\pm$ 0.12
270	0.75 $\pm$ 0.10	0.66 $\pm$ 0.13	0.56 $\pm$ 0.09
300	0.74 $\pm$ 0.09	0.64 $\pm$ 0.14	0.60 $\pm$ 0.07
360	0.64 $\pm$ 0.06	0.64 $\pm$ 0.22	0.55 $\pm$ 0.08
480	0.60 $\pm$ 0.02	0.58 $\pm$ 0.10	0.54 $\pm$ 0.08

TABLE XIV, CONTINUED

b. Carbon-14 Activity

Time of Sample	Control	Rachitic	Uremic
5	1	1	1
10	0.68±0.17	0.73±0.06	0.78±0.06
15	0.45±0.25	0.56±0.02	0.66±0.07
20	0.42±0.06	0.47±0.05	0.65±0.06
25	0.36±0.10	0.48±0.07	0.56±0.06
30	0.31±0.08	0.38±0.09	0.58±0.06
40	0.30±0.07	0.39±0.08	0.63±0.11
50	0.25±0.08	0.34±0.06	0.68±0.13
60	0.27±0.07	0.35±0.06	0.72±0.06
70	0.25±0.07	0.37±0.07	0.63±0.14
80	0.26±0.08	0.32±0.07	0.76±0.18
90	0.26±0.10	0.34±0.05	0.76±0.16
100	0.24±0.06	0.39±0.01	0.76±0.14
110	0.26±0.09	0.37±0.09	0.74±0.16
120	0.25±0.08	0.33±0.10	0.81±0.17
140	0.25±0.07	0.35±0.11	0.85±0.17
160	0.25±0.09	0.26±0.02	0.90±0.16
180	0.29±0.04	0.34±0.05	0.88±0.11
200	0.21±0.07	0.35±0.04	0.87±0.17
220	0.24±0.08	0.38±0.08	0.89±0.17
240	0.24±0.10	0.37±0.07	0.92±0.17
270	0.23±0.08	0.40±0.06	0.85±0.29
300	0.22±0.08	0.39±0.07	0.92±0.19
360	0.23±0.07	0.35±0.04	1.02±0.34
480	0.23±0.04	0.40±0.14	1.07±0.24